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DEPARTMENT OF PATHOLOGY
CYTOGENETICS LABORATORY

UNIVERSITY OF MARYLAND

July 31, 2000

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This letter is confirmation that ANITA SANOW has revised her Master's Thesis to the satisfaction of all committee members. We are pleased to accept her thesis in fulfillment of the Graduate School requirements.

Sincerely,

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Abstracts

Detection of Telomerase Activity Using Capillary Electrophoresis with Laser Induced Fluorescence (CE-LIF), Poster Presentation, University of Maryland, Graduate Student Research Day, April 2000.

OPTIMIZATION OF CAPILLARY ELECTROPHORESIS WITH LASER INDUCED FLUORESCENT (CE-LIF) DETECTION FOR THE ANALYSIS OF DOUBLE STRANDED DNA POLYMERASE CHAIN REACTION PRODUCTS FROM THE TELOMERIC REPEAT AMPLIFICATION PROTOCOL

Anita D. Sanow

Thesis Directed by: W. Edward Highsmith, Ph.D., Associate Professor, Department of Pathology, University of Maryland, Baltimore.

Telomerase is an enzyme that is found normally in mammals in embryonic cells, germ cells, and in low levels in renewal tissue such as leukocytes. Telomerase activity has been detected in 85-95 % of all tumor types tested, but is undetectable in normal somatic cells. The high prevalence of telomerase activity in tumor tissue, and its relative absence in somatic tissue, would make it ideal as a universal tumor marker, allowing earlier detection and intervention, and ultimately increasing patient survival rate. Telomerase activity has been detected in the blood of lung cancer patients with sensitivities that correlate well when compared to sensitivities obtained testing biopsied tissue specimens. The procedure currently used for detecting telomerase activity is time-consuming, cannot be automated, uses hazardous radioisotopes, and is unsuitable for high-throughput analysis.

We have developed a method using Capillary Electrophoresis with Laser Induced Fluorescent (CE-LIF) detection to analyze telomerase activity that is sensitive, does not utilize radioisotopes, can be automated, and has the potential for high-throughput testing. Experiments were performed to evaluate the effects of various parameters for the separation of TRAP assay products. A reliable separation protocol was achieved. This opens the way for accelerating the process of testing telomerase activity and elucidating its utility as a tumor marker and prognostic tool to aid the clinician in increasing the survival rate for cancer patients.

Optimization of
Capillary Electrophoresis with Laser Induced Fluorescent (CE-LIF) Detection
for the Analysis of
Double Stranded DNA Polymerase Chain Reaction Products
from the Telomeric Repeat Amplification Protocol

by

Anita D. Sanow

Thesis submitted to the faculty of the Graduate School
Of the University of Maryland in partial fulfillment
Of the requirements for the degree of
Master of Science
2000

DEDICATION

In Loving memory of my grandfather Joseph John Hrachovina,

and to my grandmother Elizabeth Rose Hrachovina who taught me many important things in life, most importantly, that the riches in life are the people you meet, the friendships you make, and the love that you share along the way. And to my husband Stephen Sanow, and my sons Nikolas and Dustin, who make life worth living.

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LIST OF ABBREVIATIONS

APS	Ammonium Persulfate
attg	Attogram (10^{-15})
BME	2-mercaptoethanol
Bp	Base Pair
CAE	Capillary Array Electrophoresis
CE	Capillary Electrophoresis
CGE	Capillary Gel Electrophoresis
cm	Centimeters
CX	Reverse primer Complimentary to the TTAGGG repeat
DNA	Deoxyribonucleic Acid
ds	Double Stranded
EOF	Electroendoosmotic Flow
HETP	Height Equivalent to a Theoretical Plate
HPLC	High Performance Liquid Chromatography
hTERT	Human Telomerase Enzyme Reverse Transcriptase
i.d.	Internal Diameter
ITAS	Internal Telomeric Repeat Standard
LIF	Laser Induced Fluorescent
mg	Milligram (10^{-3})
ml	Milliliter (10^{-3})

μg	Microgram (10^{-6})
μl	Microliter (10^{-6})
MMA	Master Mix A
MMB	Master Mix B
NSCLC	Non-Small-Cell Lung Cancer
nm	Nano-Meter (10^{-9})
PAGE	Polyacrylamide Gel Electrophoresis
PCR	Polymerase Chain Reaction
PMT	Photo Multiplier Tube
PVP	Polyvinylpyrrolidone
RNA	Riboucleic Acid
rpm	Revolutions Per Minute
SCLC	Small-Cell Lung Cancer
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
TBE	Tris Borate EDTA buffer
TRAP	Telomeric Repeat Amplification Protocol
TS	Telomerase Substrate forward primer

INTRODUCTION

Overview

Telomerase is a ribonucleoprotein enzyme that uses an internal RNA component as a template for the synthesis of DNA on the ends of chromosomes during cell replication. In mammals, telomerase is normally found only in embryonic cells, germ cells, and, in low levels, in renewal tissue such as leukocytes (Shay *et al* 1997). Most somatic cells have no telomerase and thus can undergo only a limited number of cell divisions before they senesce. Malignant cells, however, have high levels of telomerase activity, allowing these cells to divide indefinitely. Telomerase has been detected in nearly 90% of tumors of all types tested (Hiyama *et al* 1995a). All measurements of telomerase activity in tumors reported to date have used cellular material. A less invasive procedure would potentially allow the utilization of telomerase activity as a valuable screening marker and prognostic tool. A plasma assay for telomerase activity could have broad utility as a universal tumor marker.

Investigators in the Division of Human Genetics and the Greenebaum Cancer Center, UMB have shown that telomerase is detectable in the plasma of patients with active lung cancer (Strovel 1999). Further, they have obtained preliminary data from a small number of cases, showing a correlation between the plasma telomerase levels and clinical stage of disease. Based on these observations, the hypothesis that plasma telomerase activity may provide a useful marker for the potential screening, prognosis, and therapeutic monitoring of patients with a variety

of cancer types is currently being tested.

All measurements of telomerase activity, in tumor tissue or in plasma, are currently done using the Telomeric Repeat Amplification Protocol (TRAP) assay of Kim *et al* (1994). This polymerase chain reaction (PCR) based assay utilizes the PCR amplification of telomerase mediated oligonucleotide extension products, followed by detection of radioactively labeled PCR products using polyacrylamide gel electrophoresis which is analyzed by autoradiography or PhosphorImager analysis. This procedure, although useful in a research setting, is time consuming, cannot be automated, uses hazardous radioisotopes, and is unsuitable for high-throughput analysis or utilization in a clinical laboratory setting. The purpose of this project is to develop and optimize a capillary electrophoresis with laser induced fluorescence (CE-LIF) detection platform for the analysis of telomerase activity, that can be automated, has the requisite sensitivity, does not utilize radioisotopes, is user friendly, and is exportable to a clinical laboratory setting.

Chapter 1: Telomerase

Telomerase is a ribonucleoprotein that synthesizes DNA onto the ends of chromosomes, known as telomeres. These telomeric structures consist of tandemly repeated nucleotides (TTAGGG in mammals) which cap the ends of chromosomes and stabilize the DNA by guarding against enzymatic degradation and by preventing fusion of chromosomal ends. Telomeres also facilitate chromosome anchoring within the nucleus (review by Hiyama *et al* 1995b, Strovel *et al* 1998).

Telomerase functions to extend the chromosomal ends, which are partially degraded during replication. Watson defined this telomere loss during replication in 1972 and referred to it as the "end replication" problem (reviewed by Harley 1991). Telomerase is composed of several protein subunits and an internal RNA template (Kim *et al* 1994). The internal RNA template is complementary to the telomere repeat sequence TTAGGG and is referred to as human telomerase RNA or hTR (Feng *et al*1995). One of the protein subunits is a catalyst that functions in the same manner as reverse transcriptase and is referred to as human telomerase enzyme reverse transcriptase (hTERT). These two components essentially allow telomerase to act as a polymerase with its own template unit.

End Replication Problem

During replication, the newly formed strands of DNA (daughter strands) are synthesized in the 5′ to 3′ direction. While the leading strand will have continuous synthesis, the lagging strand will be synthesized in discrete segments called Okazaki fragments. An RNA primer hybridizes to the lagging strand of DNA, which allows extension of the daughter strand by DNA polymerase I. Because of the linear nature of human chromosomes, the primer eventually runs out of DNA with which to anneal, leaving a shortened 5′ end with a 3′ DNA overhang on the complementary strand that is susceptible to degradation. Telomerase acts to extend the ends of chromosomes by binding to the 3′ end of the daughter strand and catalyzing the addition of TTAGGG nucleotide repeats by copying its own RNA template (discussed by Strovel 1999).

Cellular Senescence

Since telomerase is present only in embryonic cells, germ cells, and renewal tissue, it is a natural phenomenon for the telomeres to shorten during replication in telomerase-negative cells. It has been estimated that approximately 50 base pairs are degraded per cell doubling *in vivo* (reviewed by Harley 1991). Unless telomerase activity is present, the chromosomes become shorter with each cell replication until a "critical" point, termed mortality stage 1 (M1) or the Hayflick limit, is reached, and the cell senesces. Senescent cells typically remain viable and metabolically active; only replication is stopped (reviewed by Harley 1997).

Some cells experience a transforming event that allows them to bypass the M1 stage and continue to divide, causing the chromosomes to become more and more unstable as nearly all telomeres lose their TTAGGG repeats. This results in large numbers of chromosome aberrations (predominantly dicentrics, reviewed by Harley 1991, Harley 1997). At this point, the cell enters mortality stage 2 (M2) crisis; under normal circumstances, this induces cellular senescence. Activation of the telomerase at M2 crisis is one of the events which stabilizes the chromosomes and immortalizes the cell, thereby allowing uncontrolled replication which can lead to tumor formation (reviewed by Harley 1991 and Kim 1997).

Cancer

Cancer is a general term used to describe a collection of disorders that share the common features of the transformation of normal body cells into malignant cells which proliferate with uncontrolled cell growth. There is a complex system of

biochemical reactions that govern cell differentiation, growth, life span, and ultimately cell death. Some of the systems involved in regulating cell growth are growth factors that transmit signals from one cell to another, cell surface receptors that bind with the growth factors, signal transduction molecules that activate reactions within the cell, and nuclear transcription factors. Mutations in any one of these systems can lead to the uncontrolled proliferation of malignant cells, causing the formation of tumors.

The biggest problem in the treatment of cancer is that by the time patients present with clinical symptoms, the cancer is already in advanced stages and metastases may have already occurred (Kohn and Liotta 1995). Despite improvements in the early diagnosis and treatment of cancer, many of the deaths that occur are due to metastasis that fail to respond to therapeutic treatment (Fidler 1990). Ideally, earlier detection could improve cure rates by allowing treatment when tumor burden is still small and before malignant cells have had the opportunity to spread.

Tumor Markers

With the exception of prostate-specific antigen (PSA) testing, most tumor markers to date have been unsuccessful as screening mechanisms for cancer detection. While several markers, such as CA 125 and carcinoembryonic antigen (CEA), have been useful in monitoring therapy response, they lack the sensitivity and specificity needed for screening purposes. The high prevalence of telomerase activity in tumor tissue, and its relative absence in somatic tissue, would make it

ideal as a "universal" tumor marker if a method could be developed for its detection in blood. However, no successful attempts to detect telomerase activity in blood or plasma have been reported, presumably because of the assumption that if telomerase was released into the blood stream, hTR would undergo rapid enzymatic degradation by circulating ribonucleases.

Jeffrey Strovel, Ph.D., Division of Human Genetics, UM,B, successfully modified the TRAP assay to detect telomerase activity from the blood of lung cancer patients. This modified assay successfully detected 40 out 57 patients (70 % sensitivity) with non-small-cell lung cancer (NSCLC) and 7 out of 7 patients (100% sensitivity) for small-cell lung cancer patients (SCLC). These sensitivities correlate well with biopsied tissue specimens tested by Hiyama *et al* (1995b) who detected 35 out 48 patients (73 % sensitivity) for NSCLC and 11 out of 11 patients (100 % sensitivity) for SCLC. The remarkable concordance between the positivity rates seen in tumor tissue and in blood suggests that measurement of telomerase activity in blood accurately reflects the telomerase status of the patient's tumor. Ongoing research at UM, B, is focusing on validating those observations in a larger cohort of lung cancer patients, including patients with early (stage I and II; potentially resectable) disease. Further efforts will expand the study to other types of cancer. If validated, the analysis of plasma telomerase could have widespread use as a "universal" tumor marker, with utility for screening and therapeutic monitoring of cancer. Ultimately, the test may prove valuable for the early detection of cancer in screening programs for individuals at high risk (e.g. smokers, positive family history).

TRAP Assay

The detection of telomerase activity in tumors is based on the quantitation of the enzymatic activity using the TRAP PCR assay described by Kim *et al* (1994). Strovel (1999) modified the TRAP procedure to detect telomerase activity in the blood of lung cancer patients. The procedure utilizes the radioactive isotope α-^{32}P dCTP (3000μCi/mmol) and has extended incubation times for several of the steps. Total analysis time, excluding sample preparation, protein assay, reagent preparation, and clean up time, is 24 hours. While the sample preparation and PCR procedure would remain the same, an attractive alternative to using a radioactive isotope and 13 hours of incubation for detection, would be the use of capillary electrophoresis (CE) for separation and quantitation of the PCR DNA product. CE has the capability of automating the separation and detection in a non-radioactive format that is faster and user friendly. CE technology has developed into a powerful analytical method over the past twelve years and, it has many advantages over gel based methodologies.

CHAPTER 2: Separation Techniques

Michael Faraday first formulated his Laws of Electrolysis in 1833 and 1834 based on his experiments of quantitating electrode reactions. This opened a new field of study, and for the remainder of the century, prominent scientists performed landmark experiments on small inorganic ions, enabling them to elucidate the

mechanisms of the movement of ions through an electric field, the role of electroosmosis, and the physical properties of ions based on their mobility. Leonor Michaelis coined the term "electrophoresis" in 1909 when he separated proteins based on their isoelectric points, but it was the pioneering experiments of Arne Tiselius in the 1930s that illustrated the potential use of electrophoresis for the analysis of biomolecules (for summary see Landers 1997, Camilleri 1998). Tiselius designed a system in which a sample was layered onto a buffer in an open tube. An electric field was applied across the tube and the movement of analytes was observed by photographing the tube at various times under lighting conditions which allowed visualization of zones of differential refractive index. Tiselius was able to separate the blood plasma proteins albumin, α, β, and γ-globulins, and he demonstrated that the electrophoretic mobility was related to the molecular weight of the molecules.

A major problem encountered early on was band broadening caused by heat produced in the buffer when the electric current is applied (Joule heating). Tiselius was able to reduce this effect somewhat by circulating cold water (4 $^{\circ}$C) around the electrophoretic cell. More effective techniques were developed over the next decade by using a support medium in a liquid buffer. Filter paper was used as early as 1940 with good resolution of amino acids, lipids, nucleotides, and charged sugars. The rate of migration was based on the size of the molecule and amount of current applied, while the direction of migration was based on the net charge of the ion at the pH of the particular buffer being used (Camilleri 1998).

The search for anticonvective support media continued, and starch and agarose gels were developed for the separation of peptides, proteins, and oligonucleotides. The homogeneous nature of the support media dissipates the heat in a uniform manner and the semi-solid nature of the gel decreases the movement of the heated particles, thereby reducing convection (Weinberger 1993). Polyacrylamide gels were optimized in the 1960s and have since been used for a wide variety of separation techniques for various proteins, oligonucleotides, RNA, single stranded DNA, and double stranded DNA.

Electrophoresis has had an enormous impact on research by allowing the separation of biomolecules from various complex biological samples. While polyacrylamide gel electrophoresis (PAGE) is a relatively inexpensive procedure and has become one of the most commonly used electrophoretic techniques in many laboratories today, it is also tedious, time consuming, and not very reliable in separating small charged molecules (Strege and Lagu, 1991).

High performance liquid chromatography (HPLC) was developed for the separation of smaller molecules, and has several advantages compared to PAGE. HPLC offers rapid separation and quantitation in an automated format. However, resolution decreases with larger molecules, and disposal of organic waste produced by the procedure can be expensive. This, along with the need to analyze DNA restriction fragments in a quantitative manner, led to the development of capillary electrophoresis (Strege and Lagu 1991).

CE is an extension of electrophoresis combined with the automated platform, detector technology, and chromatographic principles of HPLC (Jorgenson 1984). The earliest working model of a CE analyzer was developed by Hjerten in 1967 (reviewed by Jorgenson 1984 and Landers 1997). Recognizing the reduced thermal effects of narrow diameter tubes, Hjerten used 300 μm internal diameter (i.d.) capillaries that he rotated to reduce convection. While the rotation had little effect on heat dissipation, the mixing helped to smooth out the convection gradients (see Weinberger 1993). The use of smaller i.d. capillaries in the 1970s proved very effective in increasing heat dissipation and allowed the use of higher voltages without the need for rotation.

Due to the elimination of band broadening by Joule heating and the plug-flow characteristic of endoosmotic flow (EOF, see below), CE results in extremely high resolution for both small and large molecules. Small molecules diffuse rapidly and have high mobility, while large molecules diffuse slowly and have low mobility, allowing for high-efficiency separations (see Weinberger, 1993). The height equivalent to a theoretical plate (HETP) is used to correlate analyte peak shape and factors that effect separation in CE with HPLC. The highest HETP values can be achieved using Capillary gel electrophoresis (CGE), which has the capability of yielding theoretical plate equivalents in the millions (see Landers 1997).

Initial applications of CE to the analysis of DNA in the 1980s were based on

gel filled capillaries with successful separation of restriction fragments, and more recently, synthetic oligonucleotides, and PCR products (review, Ruiz-Martinez *et al* 1993, Heller 1998a). However, CGE has several inherent problems associated with preparing gels in small diameter capillaries. Bubbles must be avoided when filling the capillary, as they interfere with migration and can actually block the capillary entirely. Gas bubbles also form during gel polymerization and at the end of the capillary where the gel is exposed to air (Ruiz-Martinez *et al* 1993, Heller 1998a, Tagliaro *et al* 1998). Since all particles and impurities are injected along with the sample, frequent clogging may occur (Heller 1998a). The gels must be covalently bound to the capillary wall to avoid extrusion from the capillary by electroosmotic flow (EOF, see below) (Tagliaro *et al* 1998), and finally, the gels can be degraded by the alkaline pH of the buffer causing a steady decline in current (Ruiz-Martinez *et al* 1993, Heller 1998a). These problems naturally led to the exploration of gel-free buffers for use with CE.

Mobility

It is important to have a basic understanding of the principles that govern CE in order to fully appreciate its application. One of the first concepts deals with electrical conduction in fluids. While electrical current passing through an ionic solution follows Ohm's Law,

$$E=IR,$$

where E is the applied voltage; I is the current that passes through the solution; and R is the resistance of the fluid medium, the process has minor differences from solid media. Electrons carry the current in metals, where as, charged ions carry the

current in a fluid solution (see Weinberger 1993). Anions will migrate toward the anode (positive electrode) and cations toward the cathode (negative electrode) in equal quantities when voltage is applied. The charge/size ratio of the ions determine their mobility through a solution, and the size is based on molecular weight, three dimensional structure, and the degree of solvation (smaller ions are more hydrated than larger ones). Stoke's Law is used to describe the interactions of these factors:

$$f = 6\pi\eta rv,$$

where η = viscosity; r = ionic radius; v = ionic velocity. Frictional drag is produced by the ion moving through the supporting electrolyte and is directly proportional to viscosity, size, and electrophoretic velocity. Mobility of an ion through a solution can therefore be expressed by the following equation:

$$\mu = v/E = q/6\pi\eta r,$$

where q = the net charge and E = the electric field strength. Mobility is the fundamental parameter of CE, and electroosmotic flow (EOF) is an important contributing factor.

Electroosmotic Flow (EOF)

Fused silica capillaries are routinely used in CE because of their UV transparency, durability (when coated with polyimide), and zeta potential. The inner wall of the capillary carries a negative charge imparted by the ionized silanol groups (SiO^-). The charge gradient emanating from the silanol groups is referred to as the zeta potential of the capillary. The pH of the buffer determines the fraction

of silanol groups that will be ionized and a dense layer of positively charged ions forms near the inner wall. The positive charge density decreases exponentially as the distance from the wall increases (Landers 1997). When voltage is applied the cations migrate in the direction of the cathode carrying waters of hydration with them. The cohesive nature of the hydrogen bonding of water causes the entire buffer solution to be pulled toward the cathode (Landers 1997). This movement acts as a "pumping" mechanism which moves all molecules, regardless of charge, toward the detector. Therefore, the determining factor of separation would be the differences in the electrophoretic mobility of the individual analytes. As long as the EOF is adequate, the electrophoretic mobilities of each analyte will lead to discrete zones of cations, neutral molecules, and anions that pass by the detector in that order. If the EOF is too slow, diffusion of the analyte zones can occur causing band broadening, or the analytes may not reach the detector in time for analysis. If the EOF is too strong, the analytes will be swept past the detector before separation can occur. Depending on the analyte being separated, some techniques have been developed that utilize EOF to separate neutral molecules, while other techniques are used to minimize the effect. Buffer pH and capillary conditioning are extremely important factors to ensure efficient separation.

Capillary Coating

To avoid adsorption of solute to capillary walls, the capillary is usually "conditioned" by rinsing with 1 N sodium hydroxide (NaOH), followed by a 0.1 N NaOH rinse, which strips the silanol groups of any adsorbed material. The capillary is then flushed with fresh running buffer to "charge" the wall with positive

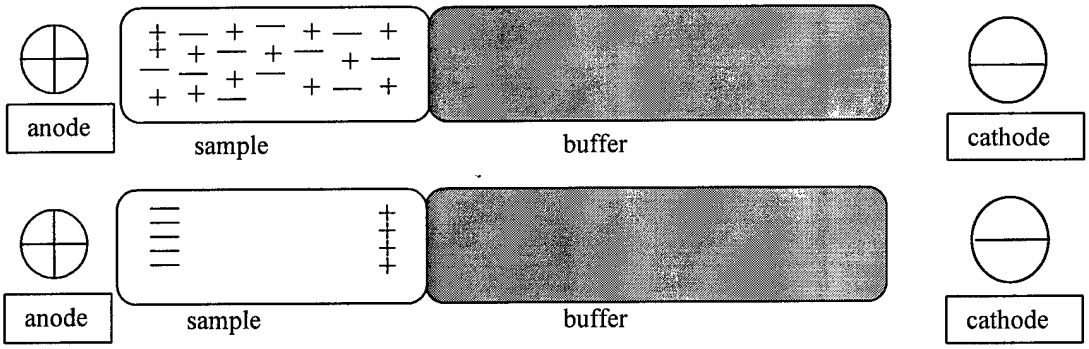
molecules, setting up the positive density gradient (Landers 1997, Weinberger 1993). This procedure ensures that the surface of the capillary is fully charged to achieve reproducible migration times (Weinberger 1993). In some instances, it is more desirable to reduce or eliminate the EOF, allowing separation of like-charged molecules. Anionic solutes, such as oligonucleotides, double stranded DNA fragments, and sodium dodecylsulfate (SDS)-treated proteins can be separated faster by eliminating EOF and reversing the polarity (- to +). Reduction of EOF can be achieved by coating the inner surface of the capillary either covalently or with adsorptive modifications. Covalent bonding usually results in permanent changes to the surface properties of the capillary and may eliminate the need for rinsing and regenerating between each analysis. Adsorptive modifications can either be permanent or replaceable, and usually involve ionic species with charges opposite that of the capillary wall. Replacement adsorption usually requires regeneration between each analysis. Ideally, the capillary coating should provide an inert surface that 1) is stable in aqueous buffer solutions in a wide pH range; 2) predictably and reproducibly modifies or eliminates EOF; 3) can be rinsed with high or low pH buffers after each analysis; and 4) remains stable for hundreds of injections (Landers 1997). The stability and reproducibility of commercially available capillaries, coated with neutral polymers, eliminates the additional requirements of developing coating techniques that fulfill the necessary requirements for the particular analyte being separated. While this makes the elimination of EOF relatively easy and separations reproducible, it also increases that cost of the capillary. Coated capillaries are four times as costly as uncoated capillaries.

Finding a polymer that "self coats" the capillary would significantly reduce the cost of testing.

Stacking

Stacking is a process that uses electrophoretic properties to concentrate the specimen prior to migration. Lower ionic strength solutions will have lower conductivity than higher ionic strength solutions, as there are fewer ions present to carry the electrical charge. As voltage is applied to a system, more resistance in the lower ionic strength solutions results in a steeper voltage drop and more rapid migration of ions. Decreasing the ionic strength of the sample compared to that of the buffer, allows faster migration in the sample fraction of the capillary (sample plug) than in the running buffer. This allows molecules with the same migration times to line up with each other (stacking), prior to entering the separation buffer (Figure 1). Stacking of the molecules concentrates like charges and allows migration at the same rate, in effect, increasing the sensitivity of small-volume samples.

Figure 1: Stacking



Polymer solutions

As previously stated, the problems inherent to gel filled capillaries led to the investigation of gel free buffer applications with CE. The ideal buffer should have separation efficiency comparable to that of classical gels, along with low viscosity for easy flushing of capillaries (Heller 1998a). One of the first effective separations of DNA fragments in solution was performed using 0.5 % methylcellulose in a poly(acrylamide) coated capillary (Strege and Lagu 1991). A 1kb DNA ladder was used to measure separation, and resolution was actually achieved between two bands separated by 11bp that normally were not resolved with conventional gel electrophoresis.

Investigation of the sieving properties of polymers soon followed and solutions were defined physically as being dilute, semidilute, or concentrated (Heller 1998a). Dilute solutions have polymer chains that are hydrodynamically isolated from each other and are regarded as having properties of a single chain. When the concentration of the polymer solution is increased, the polymer chains overlap each other and become entangled. The entangled polymers form a transient network of obstacles through which charged particle migrate, and the solution is said to be "semidilute." These semidilute polymer solutions were shown to give superior separation of double stranded DNA over dilute solutions (Heller 1998a).

This led to the investigation of entanglement properties of polymers, and an entanglement threshold, c*, was determined by measuring the viscosity of the polymer solution at different concentrations and finding the point of departure from linearity on a viscosity vs. concentration plot. The entanglement threshold is the point at which the polymer fibers start to overlap, and entanglement occurs at concentrations above this threshold. The pore size formed by this entanglement mesh is equivalent to the volume of space the individual fiber occupies, and is therefore dependent upon the concentration of the polymer. This means a polymer of varying molecular weights will maintain the same pore size, as long as long as equivalent concentrations are used (Heller 1998a). Since viscosity is a function of molecular weight, it would seem that once the threshold of a polymer is determined, choosing the smallest possible molecular weight would produce the most efficient resolution while maintaining low viscosity. However, molecular weight also affects the flexibility of the fibers, and increasing the polymer size has shown improved resolution (increased molecular weight decreases flexibility, Ruiz-Martinez *et al* 1993). The interaction of all of these factors was elucidated over the past years by the exploration of various polymer solutions for the utilization of high efficiency separations.

Linear polyacrylamide (LPA) was the first polymer to be used in which successful separation of DNA fragments was achieved (Strege and Lagu 1991). Since that time, numerous water-soluble polymers have been used, including polyacrylamide (PA) cellulose and its derivatives, polyethylene glycol (PEG),

polyvinyl alcohol (PVA), polyethylene oxide (PEO), methylcellulose (MC), hydroxyethylcellulose (HEC), hydroxypropylcellulose (HPC), hydroxypropylmethylcellulose (HPMC), and poly-N-N-dimethylacrylamide (pDMA), (reviewed by Gao and Yeung 1998, and Heller 1998b). Gao and Yeung (1998) examined the use of polyvinylpyrrolidone (PVP), a water soluble neutral polymer, that has a much lower viscosity compared to other polymer solutions with the same concentration and molecular weight. Because of the existence of hydrophilic carbonyl groups, PVP has strong hydrogen bonding with residual hydroxyl groups on the capillary wall, and the dense coating that results acts to suppress EOF. Good resolution was obtained using a pooled D1S80 ladder having a total of 27 alleles ranging from 369 to 801bp with 16bp between adjacent alleles (Gao and Yeung 1998). To achieve single base resolution, Gao and Yeung developed a method to extract the higher molecular weight (HMW) PVP from solution, and make a sieving matrix from it. PVP is readily soluble in water and alcohol, but not acetone. In a mixture of water and acetone, the higher molecular weight fraction is less soluble than the lower molecular weight fraction and will settle out of solution. The recovered high molecular weight PVP was used to make a 5 % solution in 1X TBE with 3.5 M urea. Single base resolution was achieved for 530 bases with partial separation at 439/440, 491/492, and 501/502 bases. The very low viscosity of PVP, along with the efficient EOF suppression, make it an ideal polymer for high-throughput testing. The self coating properties would allow the use of the less expensive uncoated capillaries, and it was for all of these reasons that PVP was chosen for this research.

The revolution in molecular biology, spurred by the invention of the polymerase chain reaction (PCR) in 1985 (Saiki *et al*), and considering the recent announcement of the completion of the rough draft of the sequence of the human genome, has necessitated a high throughput platform for DNA analysis. The increased sensitivity with the addition of laser induced fluorescence (LIF), and the increased speed with high-throughput capillary array analysis, made CE an invaluable technology in completing this monumental task.

SPECIFIC AIMS

The specific aim of this project is to develop a capillary electrophoresis laser induced fluorescent (CE-LIF) assay for the detection and quantitation of telomerase activity using the TRAP assay. This will be accomplished by completion of the following goals:

1. Optimization of the separation of DNA fragments by varying the concentration of sieving matrix, poly(vinylpyrrolidone) (PVP), and electrical field strength. Peak separation will be tested using a model system, on commercially available DNA ladders.
2. Determination of running conditions which minimize analysis time and optimize peak resolution for the model system. Resolution effects of buffer additives such as methanol will be evaluated, as well as stacking effects of varying ionic strength of the sample buffer.
3. Demonstration that the optimizations carried out in goals 1 and 2 result in a system capable of delivering sufficient resolution for the analysis of TRAP assay products.

Future studies will focus on determination of system linearity, sensitivity, and specificity. Ultimately, this system may prove useful for the investigation of the utility of the plasma telomerase assay as a biomarker for the detection and therapeutic monitoring of cancer, as proposed by Strovel (1999).

EXPERIMENTAL METHODS

SOLUTIONS

Bio-Rad CE dsDNA 1000

Bio-Rad CE dsNDA 1000 Fluorescent Detection and Analysis Kit , Catalog Number 148-4133. The buffer from the kit was initially used for separation of the TRAP PCR A549 cell line.

Polyvinylpyrrolidone (PVP) Sieving Buffer

A. PVP - average molecular weight 1,300,000 (Sigma-Aldrich) was dissolved in 1X TBE at concentrations from 4.9 to 7.5 %. Due to the viscosity of the buffer, reproducibility of separation between buffers prepared at different times was improved by using 5-10 ml less of 1X TBE, stirring the mixture overnight, and adding enough liquid (quantity sufficient, QS) to obtain the desired volume. This ensured accurate buffer concentrations and improved reproducibility of migration times. Once samples were loaded onto the auto-sampler, buffer was replaced after 24 hours to counteract the effects of evaporation brought on by the relatively long time it took to run each specimen.

B. High Molecular Weight PVP - The protocol developed by Gao and Yeung (1998) was used and is summarized as follows: 3 volumes of Acetone / 2-propanol (9:1 v/v) was mixed with 1 volume of 5 % PVP (w/v) in distilled water; the mixture

was shaken vigorously and allowed to stand overnight. Low molecular weight PVP is soluble in acetone/2-propanol, while the high molecular weight PVP settles out as an amorphous mass. The supernatant was discarded and the viscous layer on the bottom was poured onto a Teflon surface and allowed to dry to completion in room air (until the same weight was obtained over several days, recovery yield is ~50 %). In order to insure reproducible preparation of the running buffer, a stock solution of 10 % (w/v) high molecular weight (HMW) PVP was prepared. HMW PVP was weighed and a 10 % w/v solution in 1X TBE was prepared by overnight stirring. Working dilutions were prepared as needed by the addition of 1X TBE to the stock solution.

Detection of DNA Fragments

DNA was detected using the fluorescent dye SYBR® Green I (FMC BioProducts). The ideal fluorescent dye for separating double-stranded (ds) DNA using LIF CE should have (1) an excitation maximum of the DNA-dye complex close to that of the available laser wavelength, (2) low intrinsic fluorescence of the dye when it is not complexed to the DNA; large fluorescent enhancement upon binding to the DNA, (3) uniform binding, (4) high fluorescent quantum yield of DNA-dye complex; and (5) a large linear detection range (Schwartz and Ulfelder 1992). SYBR® Green I is a monomeric minor groove binding dye that has shown all of these characteristics as well as having a large linear detection range of dsDNA and high resolution of small dsDNA fragments (Skeidsvoll and Ueland 1995).

Protocol

1. *Staining of polyacrylamide gels:* 20 µl of 10,000X SYBR® Green I stock solution was added to 100ml of 0.5 % TBE for staining polyacrylamide gels. Gels were stained for 20 minutes and then photographed on a transilluminator at 354 nM.

2. *Preparation of sieving matrix for CE-LIF:* 100-fold dilutions were made of the 10,000X SYBR® Green I using HPLC grade dH_2O. The 100X dilutions were then added to the running buffer matrix at final concentrations of 0.1X, 5X, and 10X.

DNA Size Standards

Commercially available 100bp, 25bp and 10bp ladders from GIBCO BRL were used at 10ng/ml, and 100ng/ml concentrations. A synthetically produced T8 primer was used to verify that the PCR extension and amplification steps were working correctly. The T8 primer contains seven TTAGGG repeats that give rise to 6bp TRAP ladder fragments in the absence of telomerase.

Telomerase Positive Cell Lysates

The protocol used was modified by Strovel (1999) from Dr. Shay's laboratory of the University of Texas Southwestern Medical Center and is summarized as follows: The telomerase positive K562 cell line (ATCC # CCL-243) is a lymphoblast line from chronic myelogenous leukemia (CML) and was grown in culture medium and counted on a hemocytometer. 10^5 cells were

centrifuged, the supernatant removed and 200 µl of ice cold CHAPS buffer (Table 1) was added to the pellet, retro-pipetting to mix. The suspension was left on ice for 30 minutes and then centrifuged at 16,000 g for 20 minutes at 4 °C. 160 µl of the supernatant was collected into an Eppendorf tube and flash frozen in an ETOH-dry ice bath. The lysate contains an extract from 500 cells/µl. The telomerase activity in CHAPS buffer was stable for at least 6 months at –80 °C (Strovel 1999). This material is referred to as the "protein extract" in the remainder of the text.

Telomerase positive A549 cells (ATCC # CLL-185), which are extracted from an epithelial carcinoma of the lungs, were processed in same way with the exception of being treated with trypsin prior to counting to release cells from the bottom of the culture flask.

Table 1

LYSIS BUFFER WITH 0.5 % CHAPS store aliquoted at Room Temp (RT)		
STOCK SOLUTION	**To prepare 50 ml CHAPS Buffer, Combine:**	**FINAL. CONCENTRATION**
1 M Tris-HCL, pH 7.5	500 µl	10 mM
1 M $MgCl_2$	50 µl	1 mM
0.5 M EGTA	100 µl	1 mM
10% CHAPS	2.5 ml	0.5 % (8.13mM)
100% Glycerol	5 ml	10 %
DEPC water	QS to 50 ml	
* 14.4 M BME	*17.5 µl	5 mM
* 0.2 M AEBSF	*100 µl	0.2 mM

* Omit from the stock solution. AEBSF and BME are added to lysis solution just prior to extraction.

Telomeric Repeat Amplification Protocol (TRAP) Assay

Protocol

1. 2 µl of the protein extract was added to 23 µl of master mix A (MMA, Table 2) in thin walled PCR tubes (Perkin Elmer) and incubated at 30 °C for 20 minutes.
2. The reactions were then heated to 94 °C for 30 seconds to inactivate the telomerase.
3. Twenty-five microliters of master mix B (MMB, Table 3) were added to the reaction tubes and PCR-amplification was performed for 37 cycles at:

   94 °C for 30 seconds (denaturing)

   50 °C for 30 seconds (annealing)

   72 °C for 45 seconds (extension).

   4 °C for 96 hours (storage)

Table 2

Master Mix A (MMA) (23 µl per reaction)
0.2 µg Telomerase Substrate (TS, forward primer) (5′ – AATCCGTCGAGCAGAGTT – 3′)
20 mM Tris-HCl (pH 8.3)
1.5 mM $MgCl_2$
63 mM KCl, 0.05% Tween 20 (Sigma)
1 mM EGTA
25 µM each dNTP
0.5 µM T4g32 protein (Boehringer Mannheim - Roche)

Table 3

Master Mix B (MMB) (25 µl per reaction)
0.1 µg CX (reverse primer) (5′– CCCTTACCCTTACCCTTACCCTTA – 3′)
20 mM Tris-HCl (pH 8.3)
1.5 mM $MgCl_2$
63 mM KCl
1 mM EGTA
25 µM each dNTP
0.05 % Tween 20 (Sigma)
2.5 U Hi Fi Taq polymerase (Boehringer Mannheim - Roche)

10 % Polyacrylamide Gel

PCR products were electrophoresed on 10 % polyacrylamide gels at 300 volts for 2 hours 15 minutes. The solutions for the polyacrylamide gel are listed in Table 4.

Table 4

10 % POLYACRYLAMIDE GEL
28 ml dH_2O
2 ml 10X Tris Borate EDTA Buffer (TBE)
10 ml 40 % acrylamide
175 µl 10 % Ammonium Persulfate (APS)
25 µl TEMED

Capillary Electrophoresis (CE)

Bio-Rad BioFocus® 3000 Capillary Electrophoresis System

The Bio-Rad BioFocus® 3000 Capillary Electrophoresis System is a commercially available analyzer that features personal computer management of all functions and data with self-checking diagnostics and on-line data integration. The high voltage power supply can provide up to 30 kV of power and up to 300 µA of current. The pressure injection features both a high pressure injection of 100 psi for loading buffers and a low pressure injection for sample loading. The low sample injection uses a {pressure x time} constant (psi x sec) of 5psi. The {pressure x time} constant ensures the accuracy of injection by adjusting the time to compensate for pressure fluctuations. The analyzer has dual laser capability at 488

nm and 594 nm excitation wavelengths. The emitted light from the sample is amplified by a lens and directed to photomultiplier tubes (PMT) that generate a signal proportional to the amount of light emitted. The computer normalizes the signal based on each PMT's sensitivity and gain setting and electropherograms are generated based on this data. The argon laser at 488 nm is used for detection of the SYBR® Green I at an emission of 520 nm.

Protocol

The matrix buffer was filtered using a 0.2 μm filter (Acrodisc, Gelman Sciences), SYBR® Green I was added to the matrix buffer prior to the run and the buffer was centrifuged for 3 minutes at 13,000 rpm to degas the solution. Matrix buffer was injected into a 24 cm effective length capillary under high pressure for three minutes. The capillary ends were dipped in dH_2O and methanol in between injections to prevent carryover. The specimen was injected at low pressure, reverse polarity (- to +) was used, and voltage was applied. Both coated and uncoated capillaries were used and capillaries were rinsed with dH_2O for 10 minutes at the end of each run to prevent capillary clogging.

Telomeric Repeat Amplification Protocol (TRAP) Assay

The protocol developed by Strovel (1999) was used and is summarized as follows:

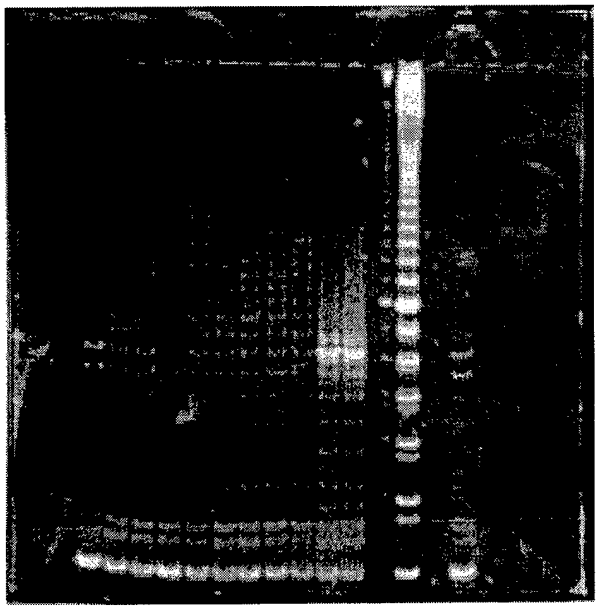
Protein extract was added to MMA (Table 2) and incubated for 20 minutes at 30 °C. During this time, telomerase added TTAGGG repeats to the 18mer oligonucleotide Telomerase Substrate (TS) forward primer, pausing after each six base pair addition, forming a six base pair ladder. The mixture was then heated to 94 °C to inactivate the telomerase. MMB (Table 3), containing the 24mer oligonucleotide reverse CX primer, was then added to the reaction. The CX reverse primer is complementary to the TTAGGG repeats added to the forward primer by telomerase. PCR amplification was performed at 94 °C for 30 seconds for denaturing; 50 °C for 30 seconds for annealing; and 72 °C for 45 seconds for extension, for 37 cycles. Amplification occurred only if telomerase was present. Ten attograms (10^{-15}) per reaction of **I**nternal **T**elomerase **A**mplification **S**tandard (**ITAS**) was added to Master Mix B (Table 3) when quantification for the TRAP ladder was required and for peak identification purposes. The ITAS is a 150 base pair DNA fragment that has binding sites for both the forward TS primer and the reverse CX primer. Competitive amplification occured when telomerase was present, with a decrease in the amount of ITAS amplified as telomerase levels increased.

RESULTS

Specific Aim 1: Optimization of the separation of DNA fragments using commercially available ladders.

Because the ultimate goal of this study is to optimally separate TRAP assay products using CE instead of the traditional gel electrophoresis, initial experiments were performed in which the two separation methods were compared. The standard TRAP assay was performed using fluorescent primers instead of radioisotopes on telomerase positive A549 cell lysates, and PCR products were subjected to gel electrophoresis. Results in Figure 2 demonstrate a clear separation of bands, representing DNA fragments differing by 6bp.

Figure 2



TRAP Assay of A549 cell lysates of increasing concentrations separated on 10 % polyacrylamide gel at 275 volts for 2 hours 45 minutes. Gel was stained with SYBR® Green I in 0.5 X TBE. Blanks are run in the first two lanes, and a 10bp ladder was run in lane # 16.

The same TRAP PCR products were then subjected to CE, under varying conditions of buffer and voltage:

a. A commercially prepared buffer, CE dsDNA 1000 kit (Bio-Rad) was used at 2.5 kV, specimen was injected at 5 (psi x sec) (Figure 3a).
b. Specimens were run under denaturing conditions using 3.5 M urea in 5 % PVP at 6.0 kV, specimen was injected at 5 (psi x sec) (Figure 3b).

NOTE: All CE-LIF separations were performed using the Bio-Rad BioFocus 3000 analyzer, and were run using reverse polarity (- to +) with a 24 cm x 50μm i.d. capillary. Both coated and non-coated capillaries were used, with no detectable difference in separation between the two when PVP was used as the buffer matrix.

Polyvinylpyrollydone (PVP) is a water soluble neutral polymer that has a much lower viscosity compared to other polymer solutions with the same concentrations and molecular weight. It also coats the capillary wall, eliminating electroosmotic flow (EOF), allowing the use of less expensive uncoated capillaries. Urea denatures the DNA, forming single stranded fragments, changing the electrophoretic migration. Resolution is based on the distance separating two peaks and there should be a peak for each band detected by gel electrophoresis. Results in Figures 2 and 3 demonstrate that the TRAP PCR was successful, but the products were not separated under either of these conditions, indicating the sieving pores are too big to separate DNA fragments of this size.

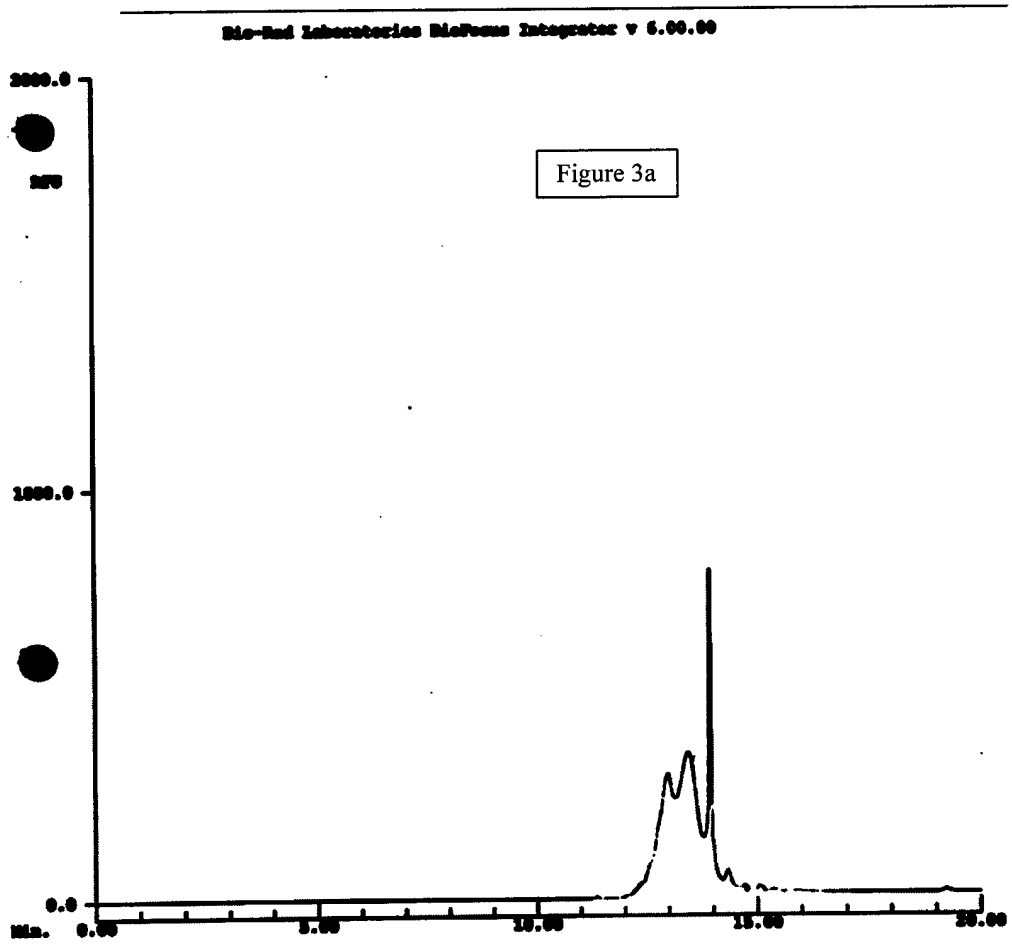


Figure 3a

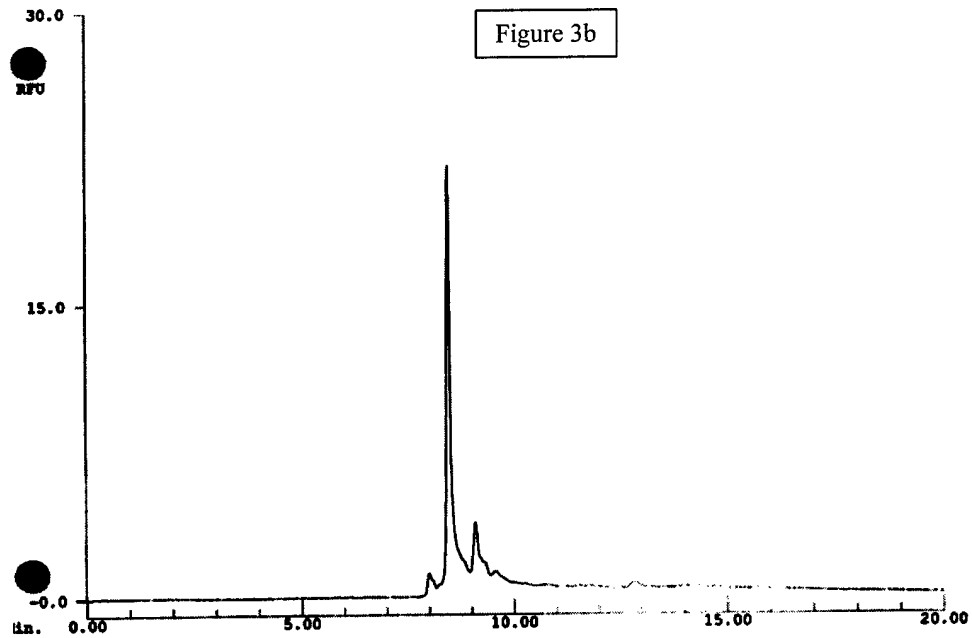


Figure 3b

Figure 3a: TRAP PCR product of A549 cell lysate with fluorescein labeled primers, separated using the CE dsDNA 1000 (Bio-Rad) Fluorescent Detection and Analysis Kit sieving matrix, run at 2.5 kV.
Figure 3b: TRAP PCR product of A 549 cell lysate with fluorescein labeled primers using 5 % PVP in 1X TBE with 3.5 M urea, run at 6.0 kV.

In order to determine the effective resolution of this sieving matrix, commercially available DNA ladders with larger DNA fragments were used instead of TRAP assay products to optimize conditions for CE separation. Furthermore, sensitivity was increased and the experimental procedure was streamlined by the use of SYBR® Green I dye (Molecular Probes, Eugene, OR). This allowed separation to be performed under non-denaturing conditions and eliminated the need to incorporate fluorescence into the PCR product. SYBR® Green I was added to 5 % PVP / 1X TBE buffer without urea. Electrophoresis was performed using a 100bp ladder at 6kV (Figure 4a). Good peak resolution was obtained with the smaller DNA fragments (100bp–500bp), however, separation did not occur with larger fragments. Therefore, the voltage was reduced to 1 kV to allow more migration time, and clear resolution was obtained at a 25bp level (Figure 4b).

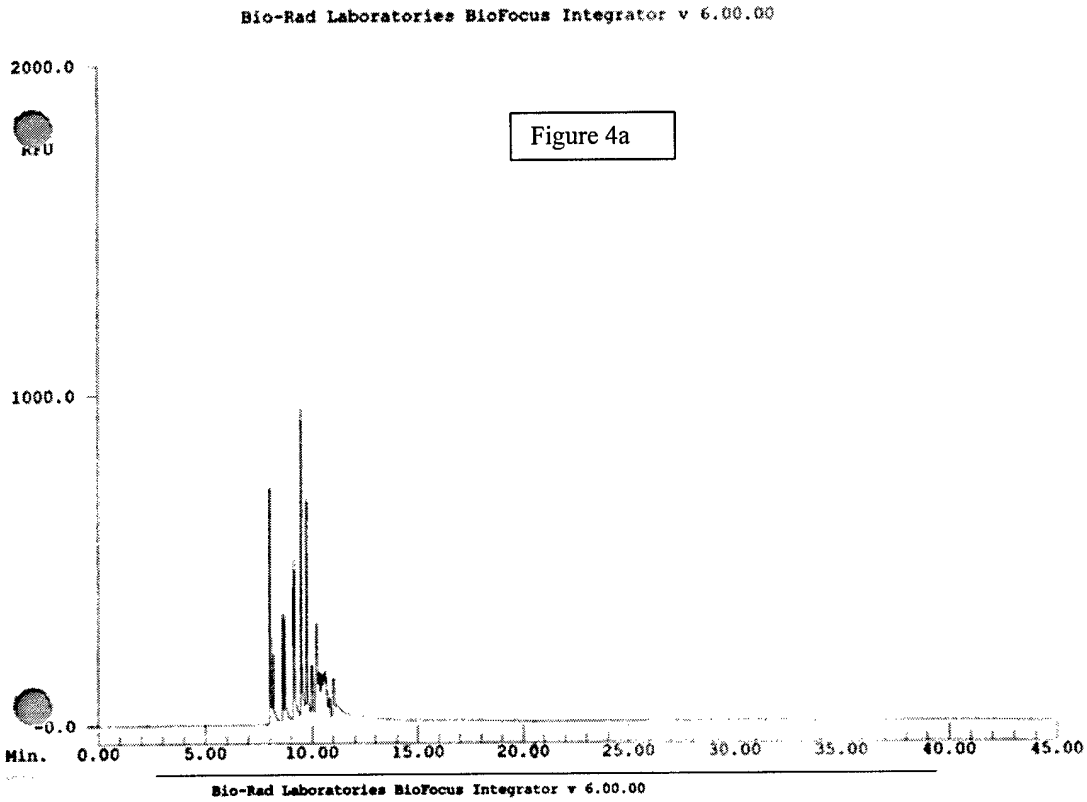


Figure 4a

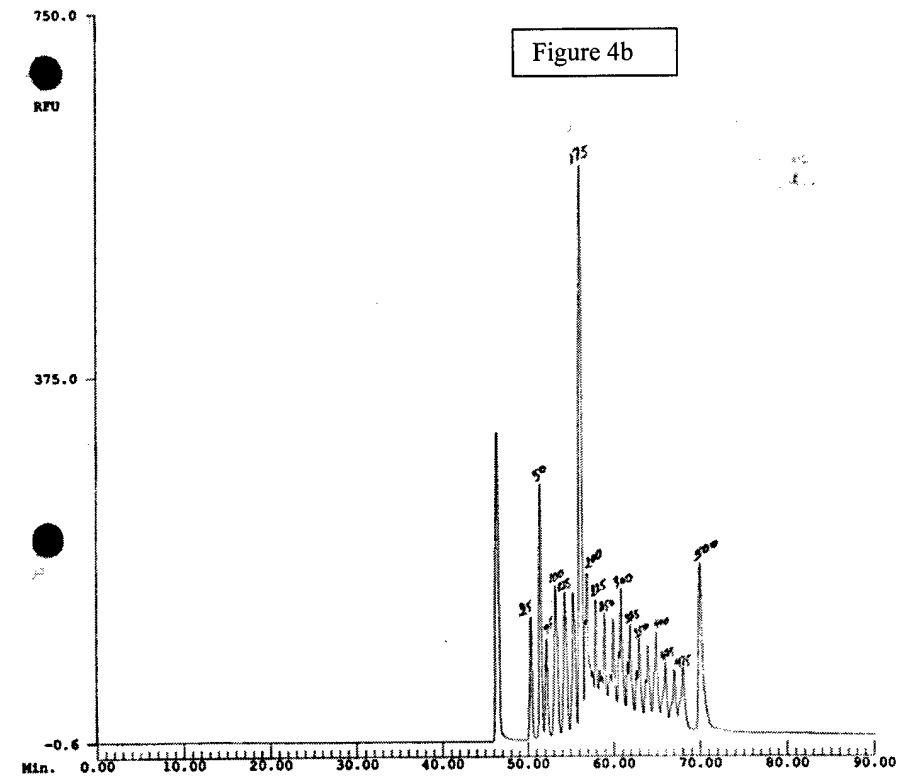


Figure 4b

Figure 4a: 100bp DNA ladder separated in 5 % PVP/1X TBE with SYBR® Green, a 6.0 kV.
Figure 4b: 25bp DNA ladder separated in 5 % PVP/1X TBE with SYBR® Green at 1.0 kV.

Separation of DNA fragments was evaluated at different sieving matrix concentrations in order to evaluate the effect of increased concentration on peak resolution. PVP concentrations of 5 %, 6%, and 7.5 % were used to separate both 25bp and 10bp ladders. Efficient separation was not achieved for the 10bp ladder at 5 % or 6 % concentrations (data not shown), and separation of both DNA ladders at 7.5 % PVP are shown in Figure 5a and 5b. As the PVP concentration was increased from 5-7.5 %, the viscosity also increased, causing problems with buffer movement through the capillary, and consequently decreasing separation efficiency.

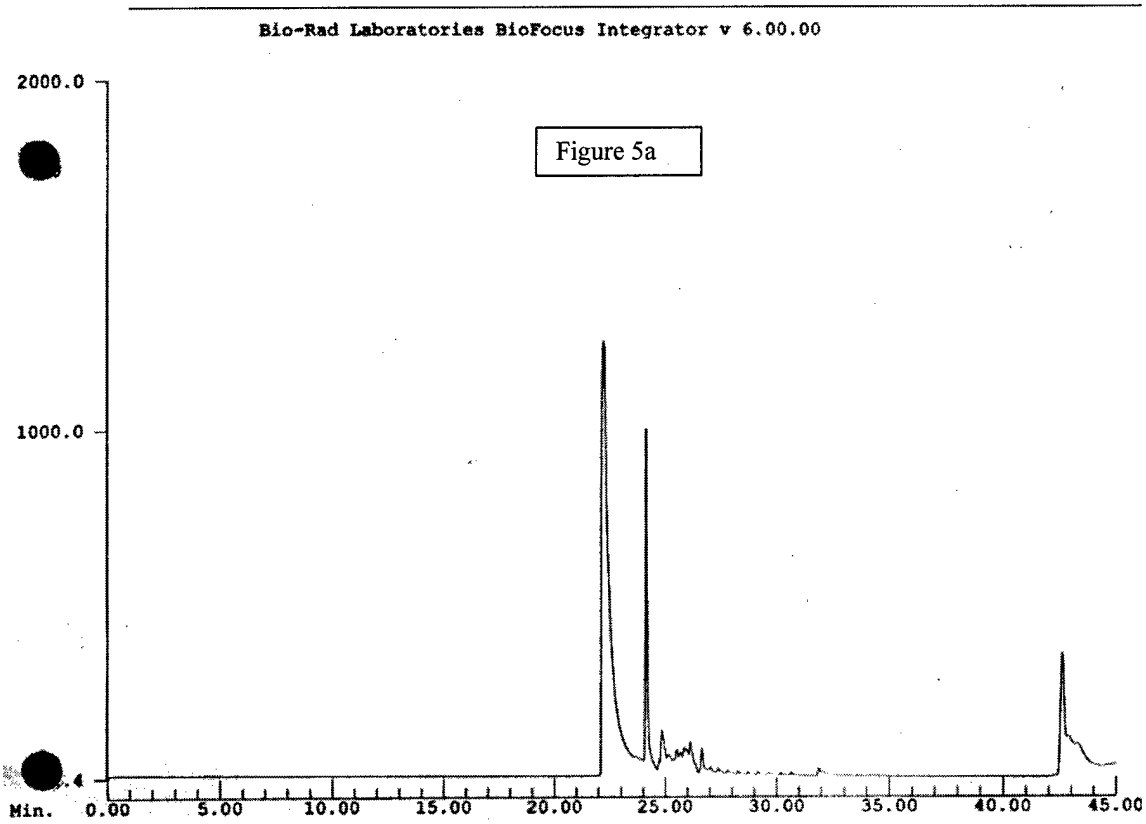


Figure 5a

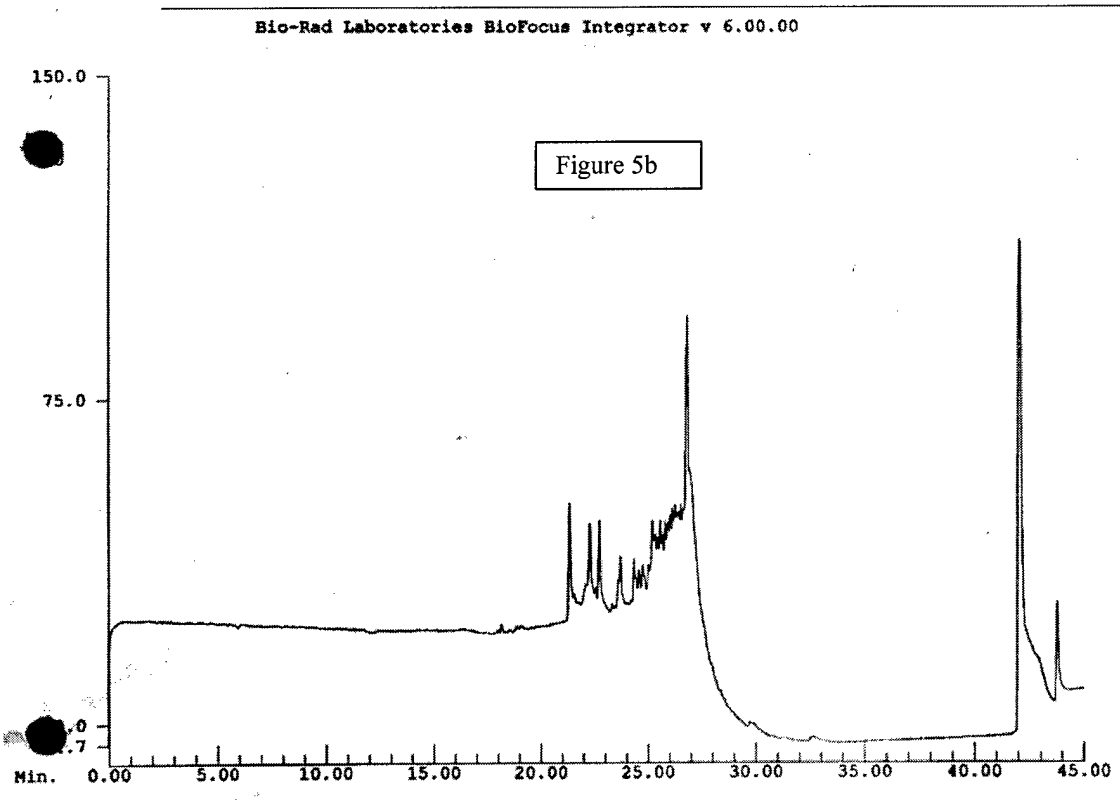


Figure 5b

Figure 5a: 25bp ladder separated in 7..5 % PVP/1X TBE with SYBR® Green at 1.0 kV

Figure 5b: 10bp ladder separated in 7..5 % PVP/1X TBE with SYBR® Green at 1.0 kV.

Gao and Yeung (1998) used a technique to fractionate high molecular weight (HMW) PVP from low molecular weight PVP (see Experimental Methods). Use of the HMW PVP for a sieving matrix reduces the viscosity of the buffer at increased concentrations. In order to determine separation effects of the HMW PVP, experiments were performed using a buffer matrix of 5 %. Resolution was obtained at a 10bp ladder level (Figure 6). Increased voltage causes faster migration times, narrowing the peak width. In order to determine the effects of increased voltage, separation of the 10bp ladder was evaluated at 1.0, 1.5, and 2.0 kV, using 5 % HMW PVP. Results show that increasing the voltage decreases the run time by more than half while maintaining good peak resolution (Figures 6a-c). However, decreasing the distance between the peaks also decreases the resolution of smaller DNA fragments, and for this reason experiments were continued at 1.0 kV.

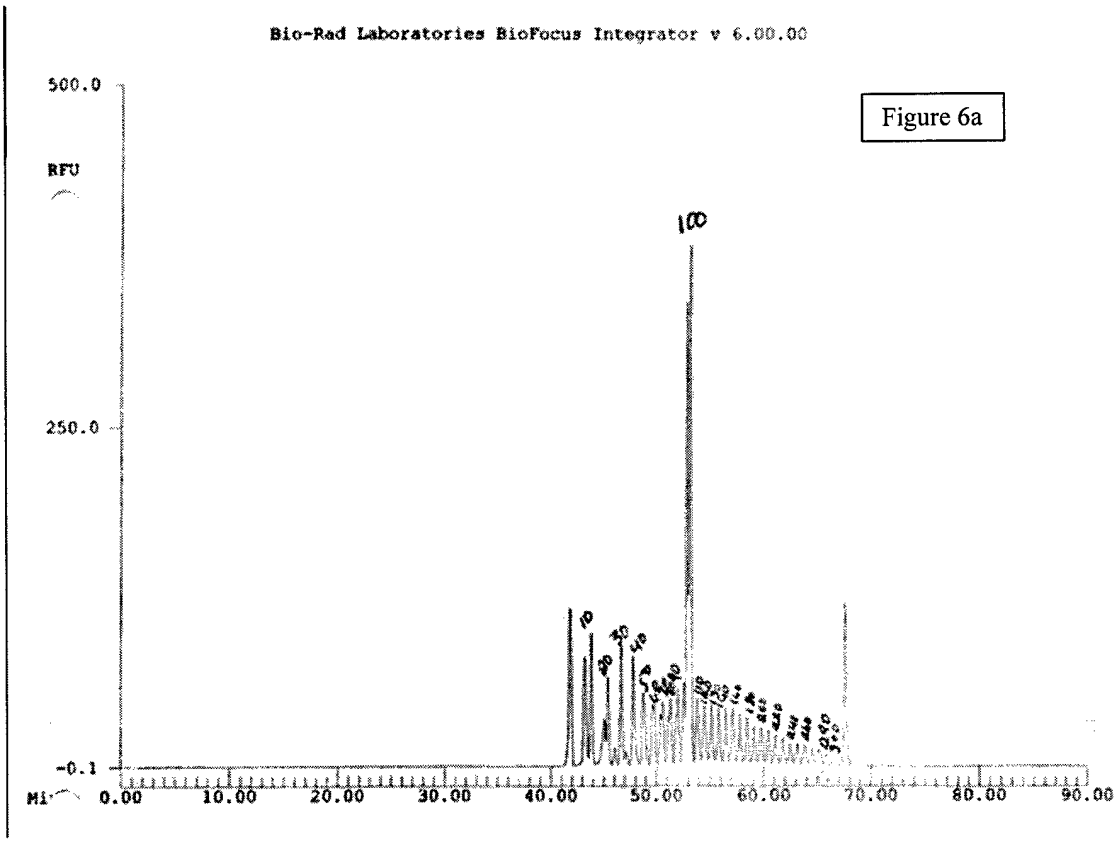


Figure 6a

Figure 6a: 10bp DNA ladder separated in 5 % HMW PVP/1X TBE with SYBR® Green at 1.0 kV.

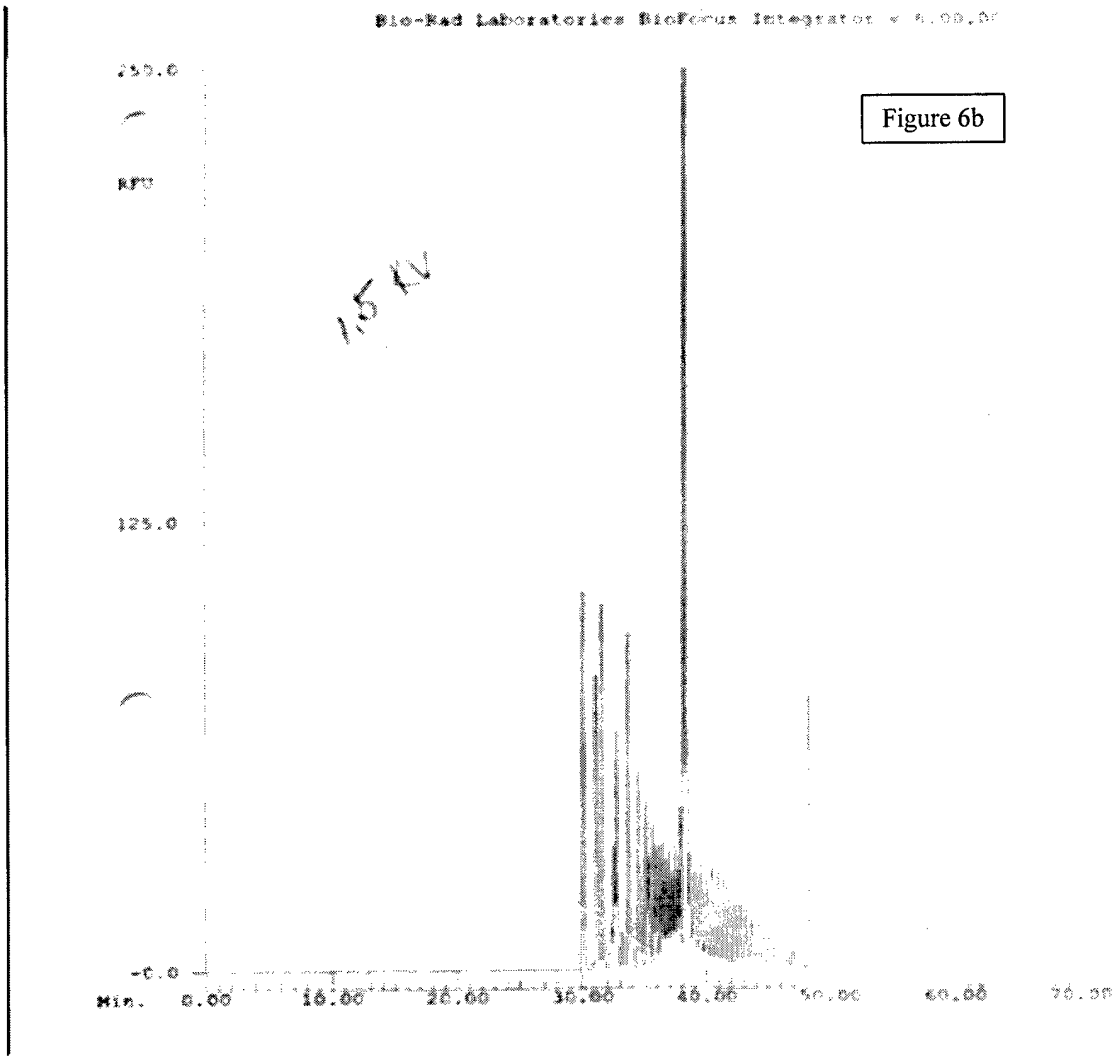


Figure 6b

Figure 6b: 10bp DNA ladder separated in 5 % HMW PVP/1X TBE with SYBR® Green at 1.5 kV.

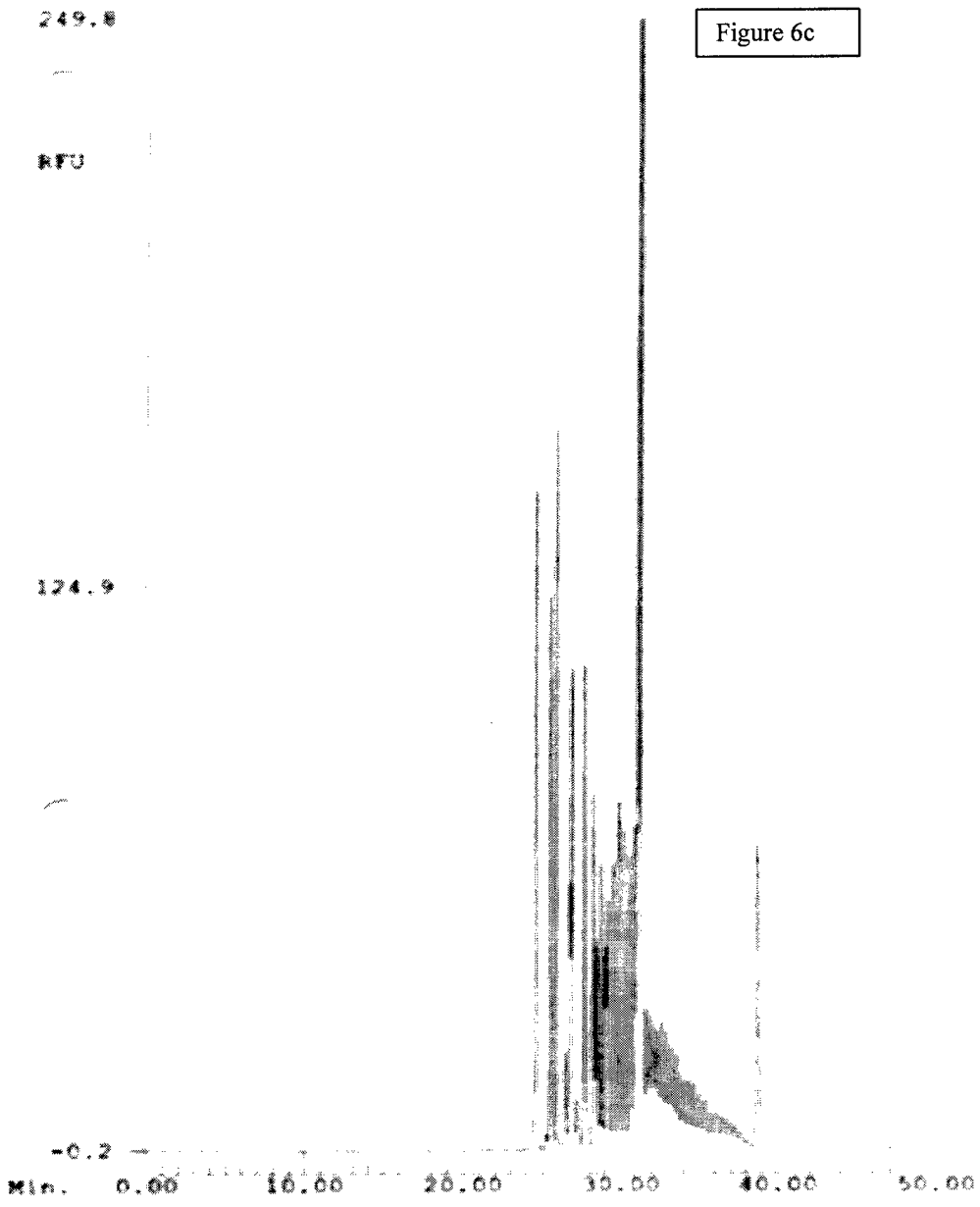


Figure 6c

Figure 6c: 10bp DNA ladder separated in 5 % HMW PVP/1X TBE with SYBR® Green at 2.0 kV.

In order to increase the distance between the peaks and allow for separation of smaller DNA fragments at a 6bp level, experiments were performed using HMW PVP at a concentration of 6 %. This resulted in increased peak resolution of the 10bp ladder (Figure 7a). These conditions were also used to separate TRAP PCR products amplified using the synthetic primer T8, which yields DNA fragments differing by 6bp in the absence of telomerase. Results from Figure 7b demonstrate good separation at the 6bp level.

Taken together, these results demonstrate that optimal conditions for separating TRAP assay products were determined to be 6 % HMW PVP at 1.0 kV.

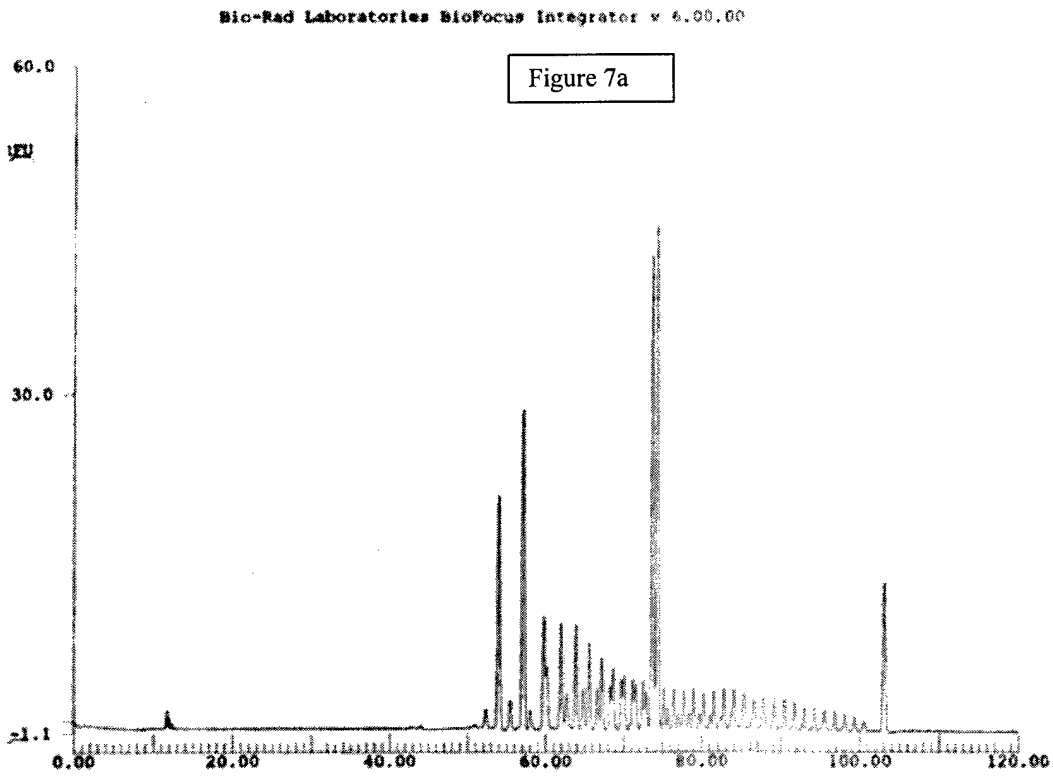


Figure 7a

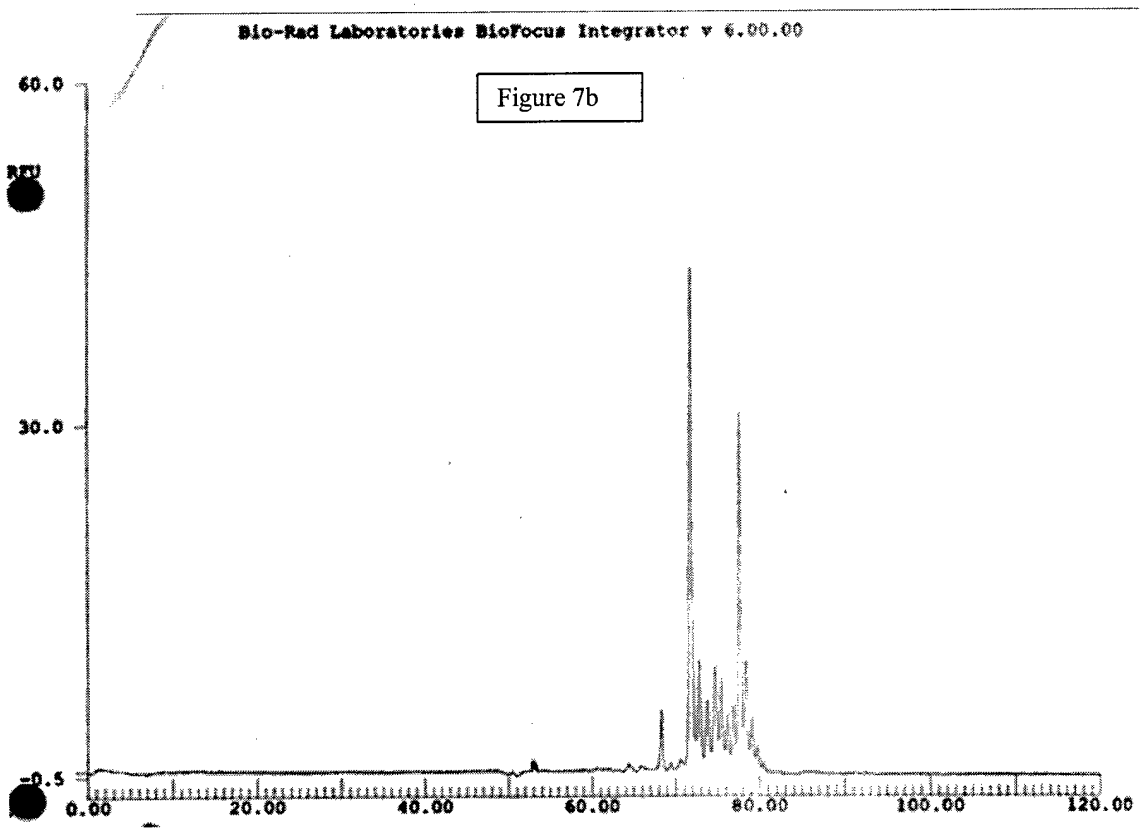


Figure 7b

Figure 7: 6 % HMW PVP/1X TBE with SYBR® Green at 1.0 kV
Figure 7a: 10bp ladder
Figure 7b: TRAP PCR of T8 primer

Specific Aim 2: Determination of conditions which minimize analysis time and optimize peak resolution.

Buffer additive

Methanol, when added to the electrophoresis buffer, serves to increase the viscosity of the buffer. In order to evaluate the effects of this additive on CE, experiments were performed in which 5 %, 10 %, and 20 % methanol was added to 6 % HMW PVP. Results show an increase in peak resolution with the addition of 10 % methanol (Figure 8a) when compared to 6 % HMW PVP without methanol (Figure 8b). The addition of methanol also slightly decreased the migration time.

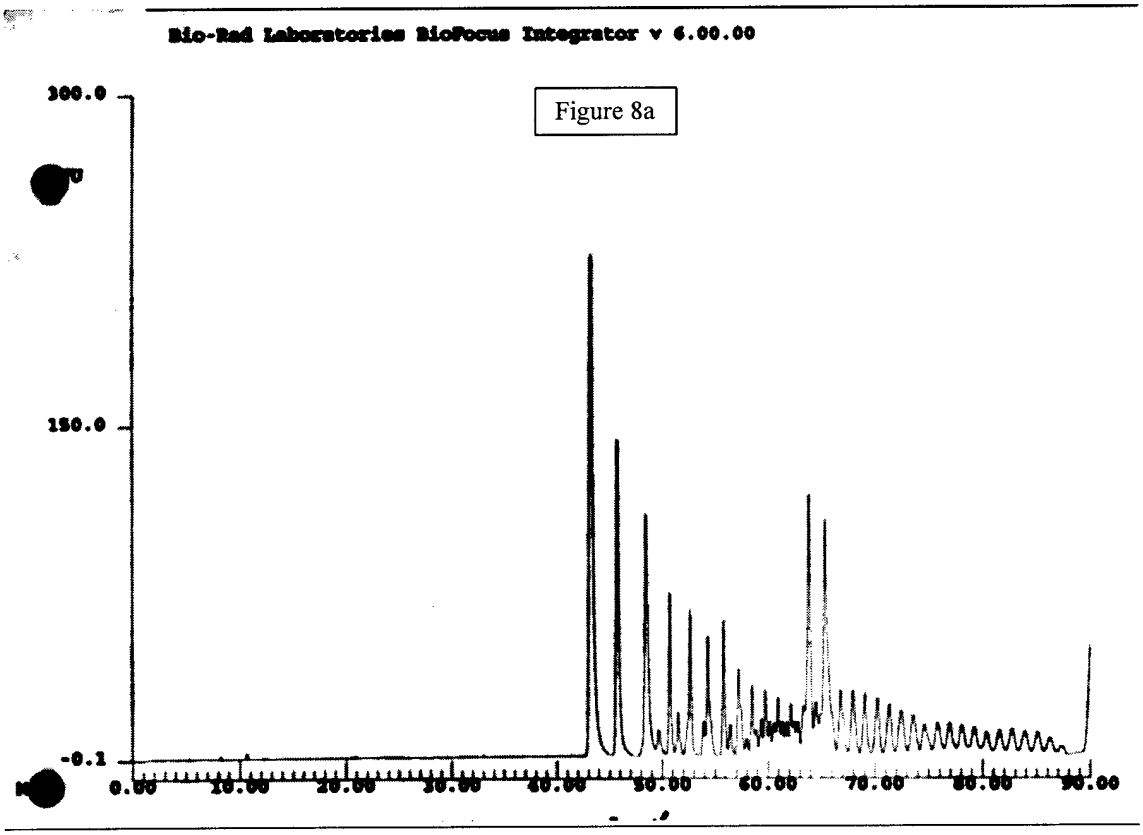


Figure 8a: 6 % HMW PVP/10 % MethanolX TBE with SYBR® Green at 1.0 kV 10bp DNA ladder

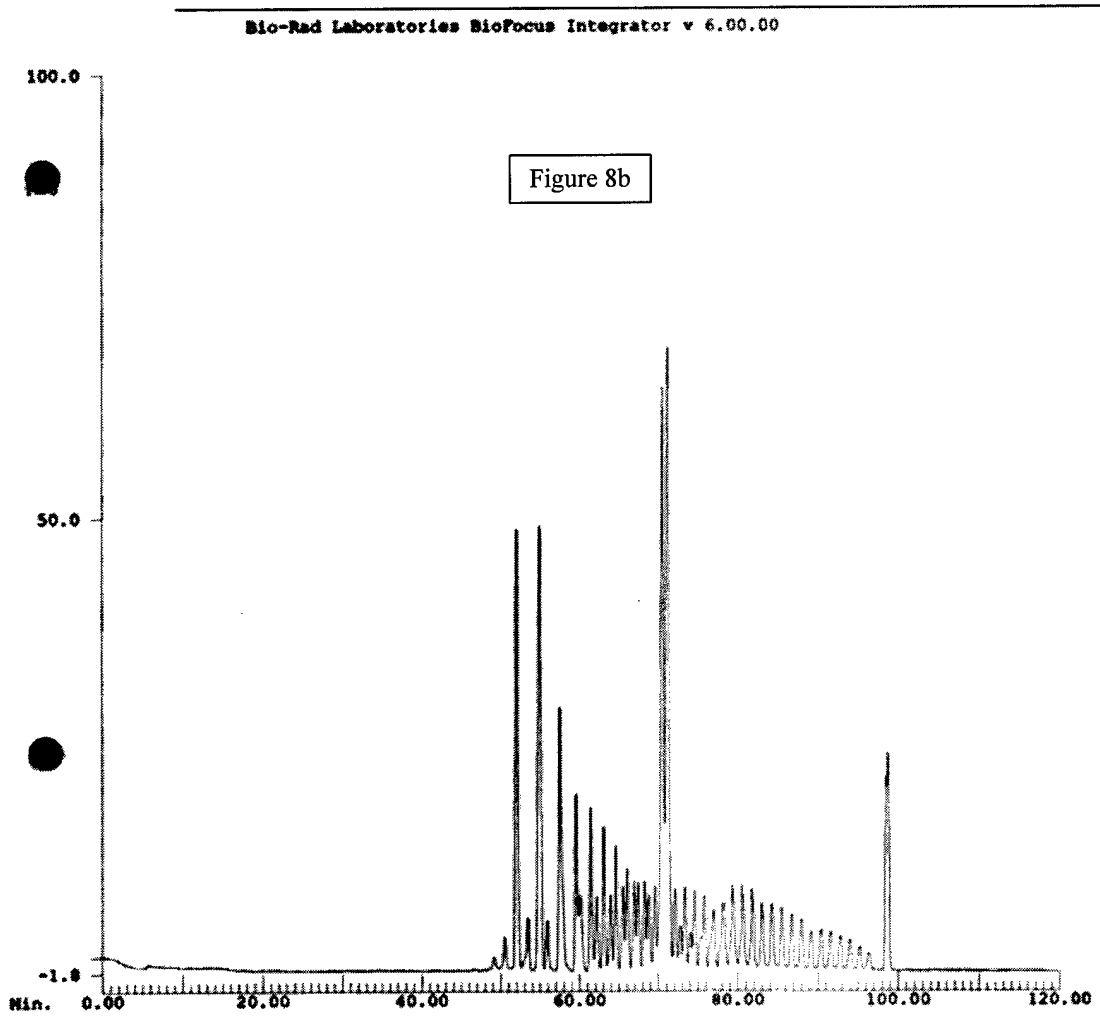
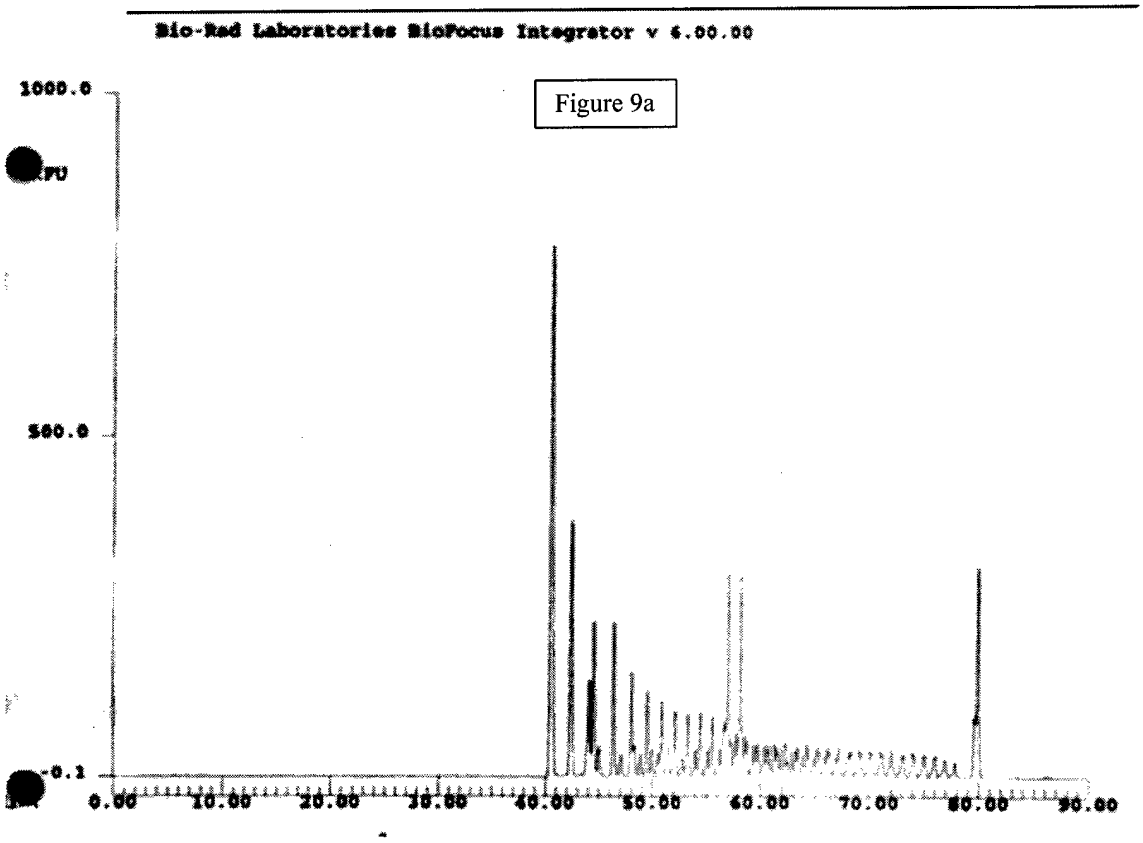


Figure 8b

Figure 8a: 6 % HMW PVP/lX TBE with SYBR® Green at 1.0 kV
10bp DNA ladder

Stacking

Lower ionic strength solutions will have lower conductivity than higher ionic strength solutions, as there are fewer ions present to carry the electrical charge. As voltage is applied to a system, higher resistance in the lower ionic strength solution results in a steeper voltage drop and more rapid migration of ions. The ionic strength of 1X TBE is 189 meq/L and that of the PCR buffer is approximately 53 meq/L. Stacking typically is optimal when the ionic strength of the sample is approximately 10 % of the running buffer. In order to achieve this, the TRAP PCR products were diluted X5 with distilled water. The apparent paradox of achieving greater sensitivity using a diluted sample is solved by realizing 1) the PCR products are concentrated in a thin zone, resulting in sharper, narrower peaks, and 2) a much larger sample can be injected onto the capillary. This, in effect, concentrates the specimen, allowing molecules with the same migration times to line up with each other (stacking) prior to entering the sieving matrix. In order to evaluate stacking effects, experiments were performed using a 10bp ladder and varying the specimen injection times from 10 (psi x sec) to 100 (psi x sec). Increasing the sample injection time lengthens the sample plug, allowing more time for a greater stacking effect. Representative electropherograms are shown in Figure 9a-9d. Results show a sensitivity increase of 1000 fold, exceeding the maximum detection limit of the detector (Figures 9c –9d).



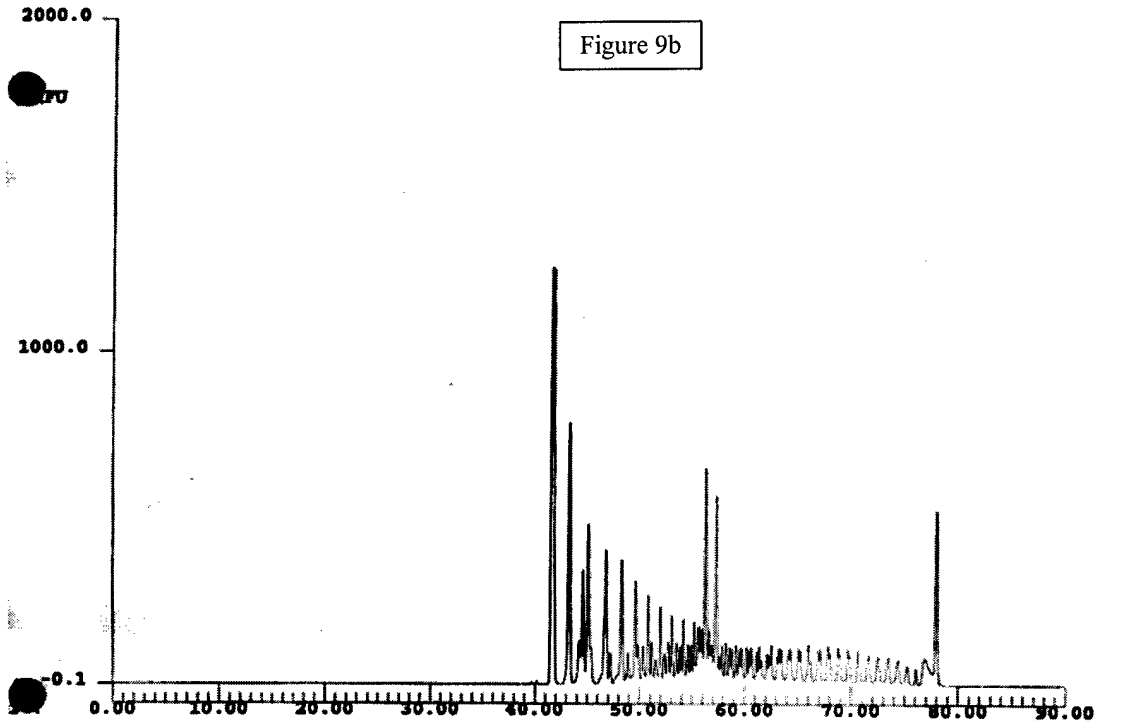


Figure 9: 6 % HMW PVP/10 % MethanolX TBE with SYBR® Green at 1.5 kV 10bp DNA ladder

Figure 9a: 20 (psi x sec)

Figure 9b: 50 (psi x sec)

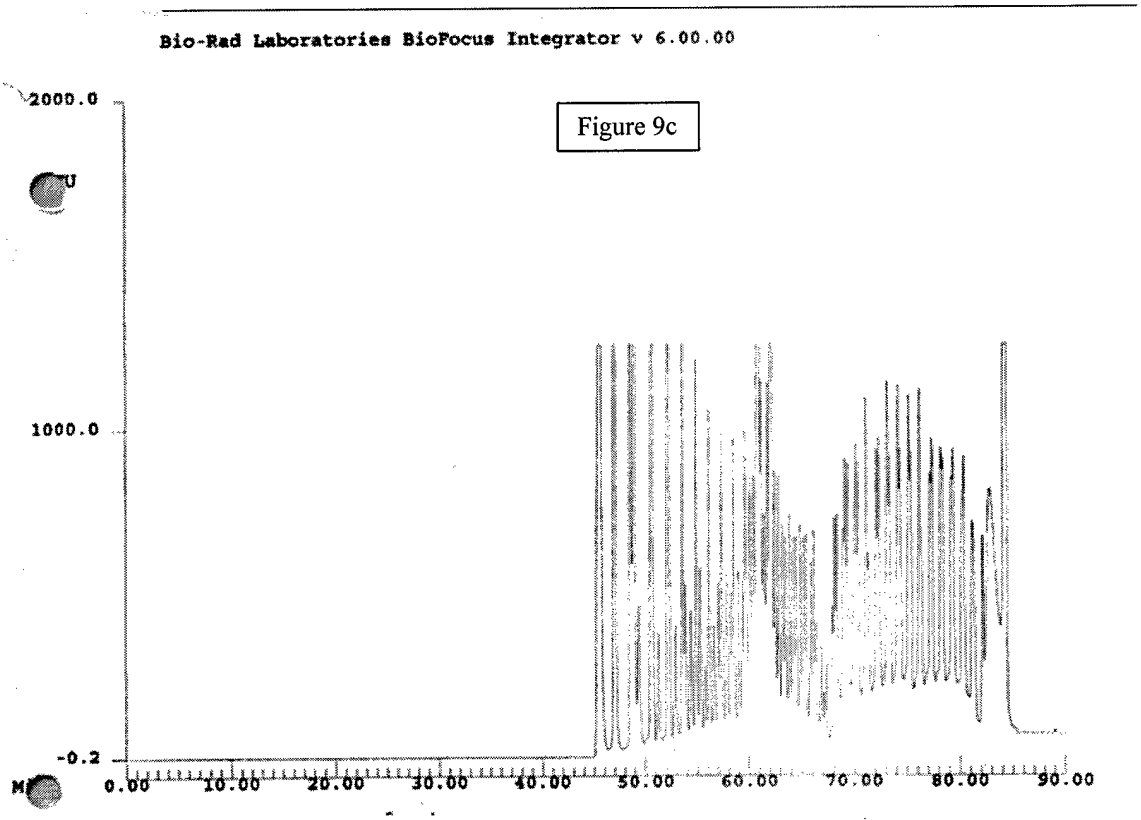


Figure 9c

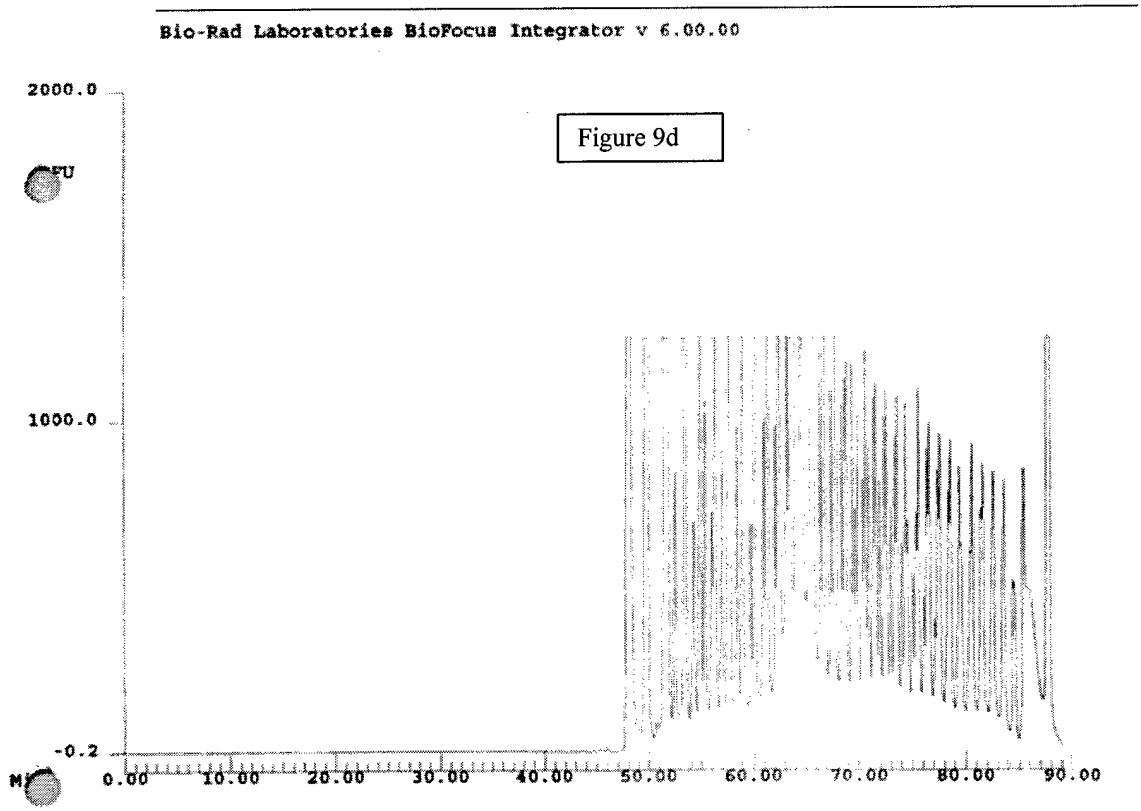


Figure 9d

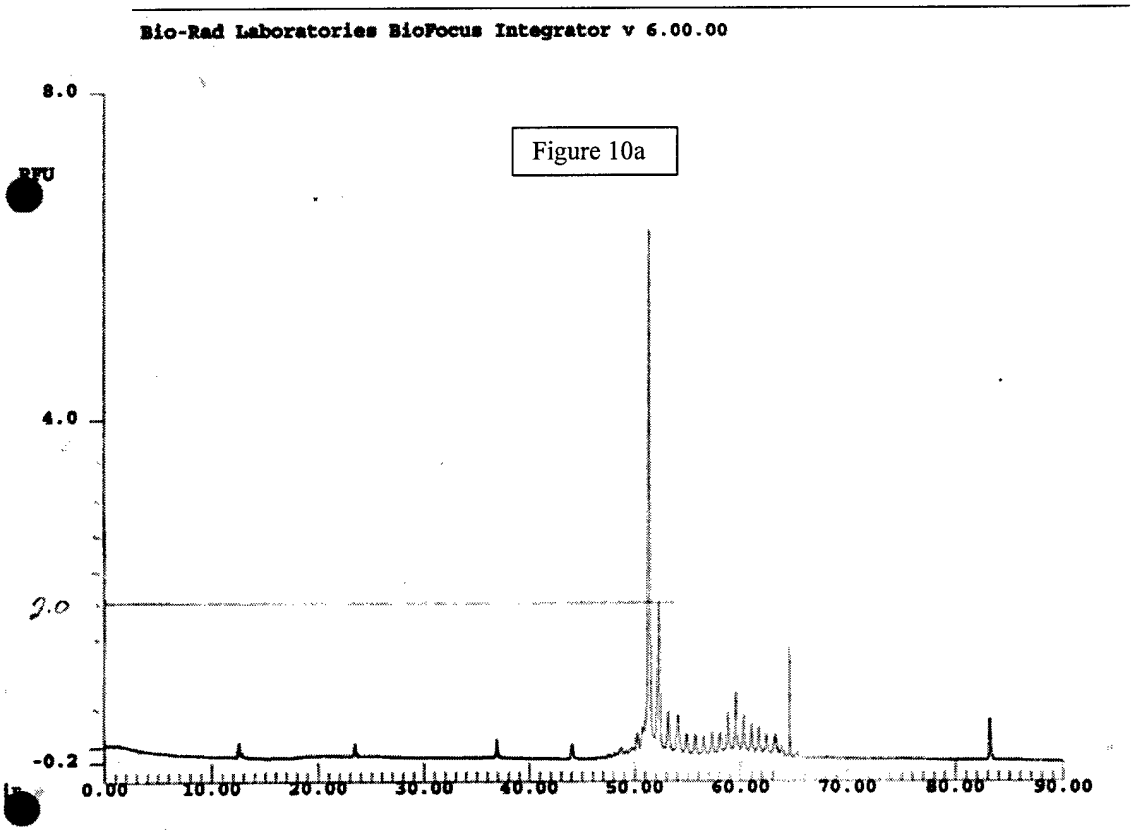
Figure 9: 6 % HMW PVP/10 % MethanolX TBE with SYBR® Green at 1.5 kV
10bp DNA ladder

Figure 9c: 70 (psi x sec)

Figure 9d: 100 (psi x sec)

To reduce the signal into the detectable range, the SYBR® Green I concentration was decreased from 10X to 5X, and experiments were again performed to test stacking effects on TRAP PCR products. Results show a two fold increase in sensitivity between 80 and 100 (psi x sec), (Figure 10a-10c). The 10pb ladder was at a much higher concentration than the TRAP PCR products, which would account for the significant difference in stacking effects.

Based on these results, optimum conditions for peak resolution with minimum analysis time were obtained with 6 % HMW PVP / 10% methanol, using 100 (psi x sec) injection times at 1.5 kV.



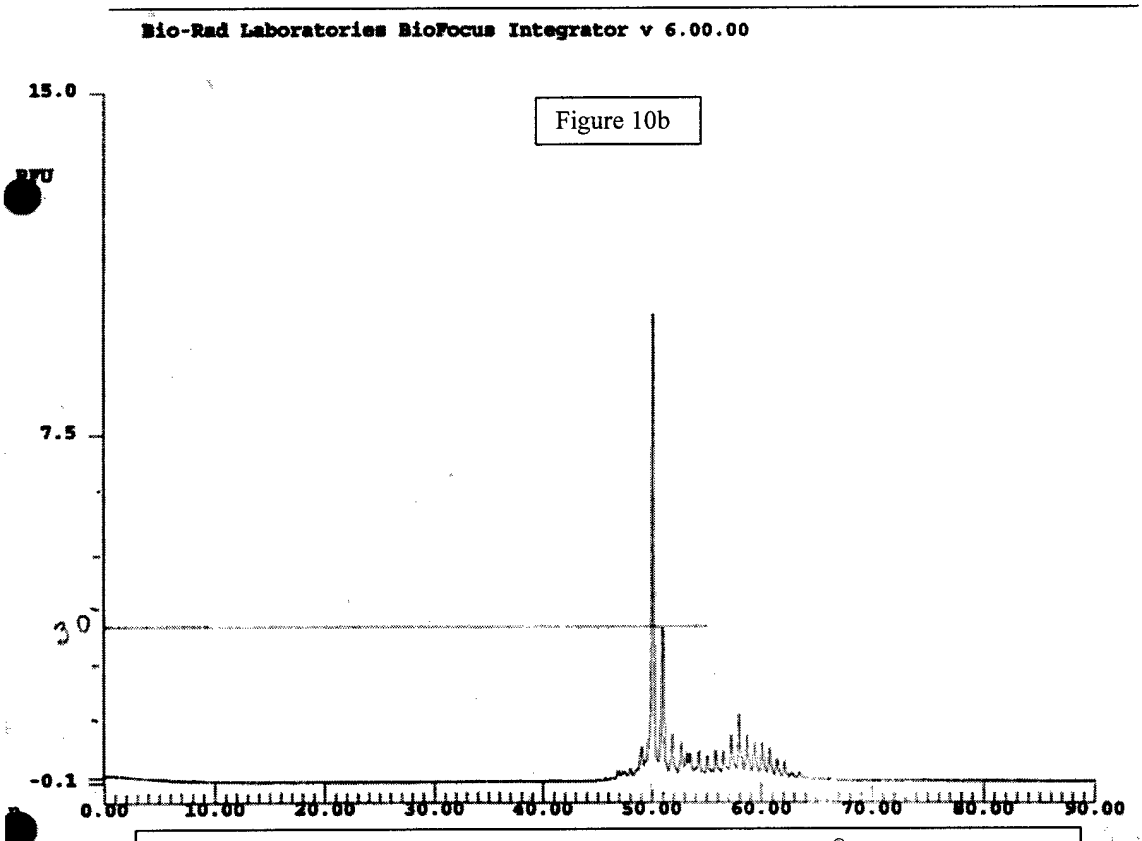


Figure 10: 6 % HMW PVP/10 % Methanol /X TBE with SYBR® Green at 1.5 kV TRAP PCR K562 25 cell lysate

Figure 10a: 80 (psi x sec)
Figure 10b: 90 (psi x sec)

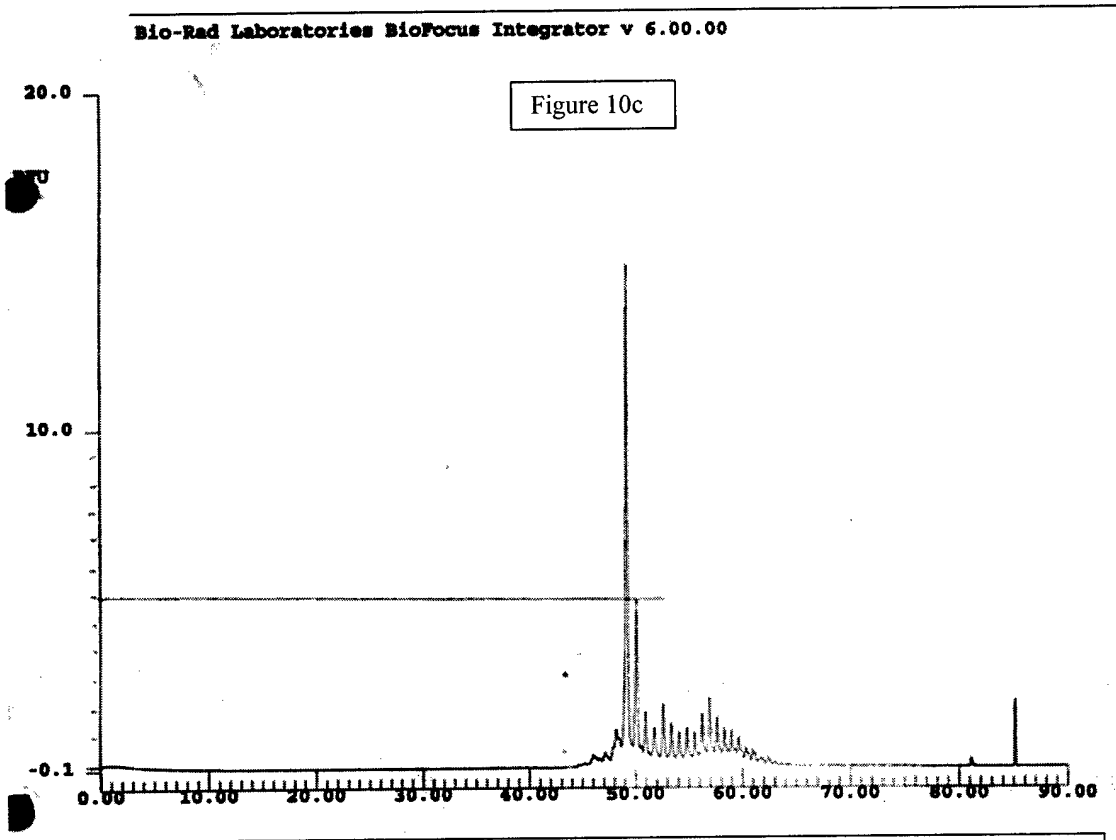


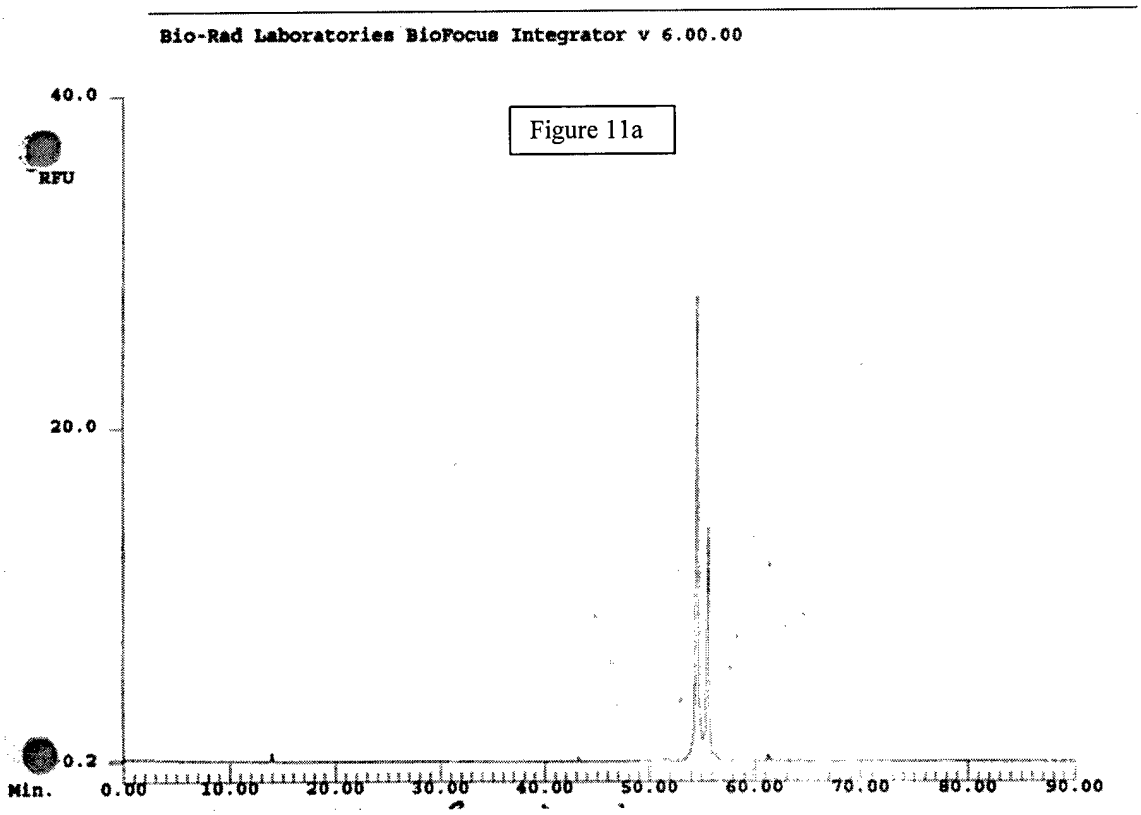
Figure 10: 6 % HMW PVP/10 % Methanol /X TBE with SYBR® Green at 1.5 kV TRAP PCR K562 25 cell lysate

Figure 10c: 100 (psi x sec)

3. Application of conditions defined in Specific Aims 1 and 2 to the separation of TRAP assay products.

In order to quantitate telomerase activity from the TRAP assay, an internal telomerase assay standard (ITAS) of known concentration is added to master mix B prior to amplification (see Experimental Methods). Competitive PCR occurs, and the amount of 6bp fragments detected is proportional to telomerase activity, while the amount of ITAS amplified is inversely proportional to telomerase activity. A primer-dimer fragment also forms during the PCR reaction. In order to quantitate TRAP PCR products following CE, the locations of both ITAS and primer-dimer fragments with respect to the 6pb ladders must be determined. Experiments were performed using 6 % HMW PVP / 10 % methanol with 100 (psi x sec) injection at 1.5 kV under the following conditions:

a. dH_2O was used for the sample blank, yielding a primer-dimer peak only (Figure 11a).
b. ITAS was amplified using dH_2O as sample producing both primer-dimer and ITAS peaks (Figure 11b).



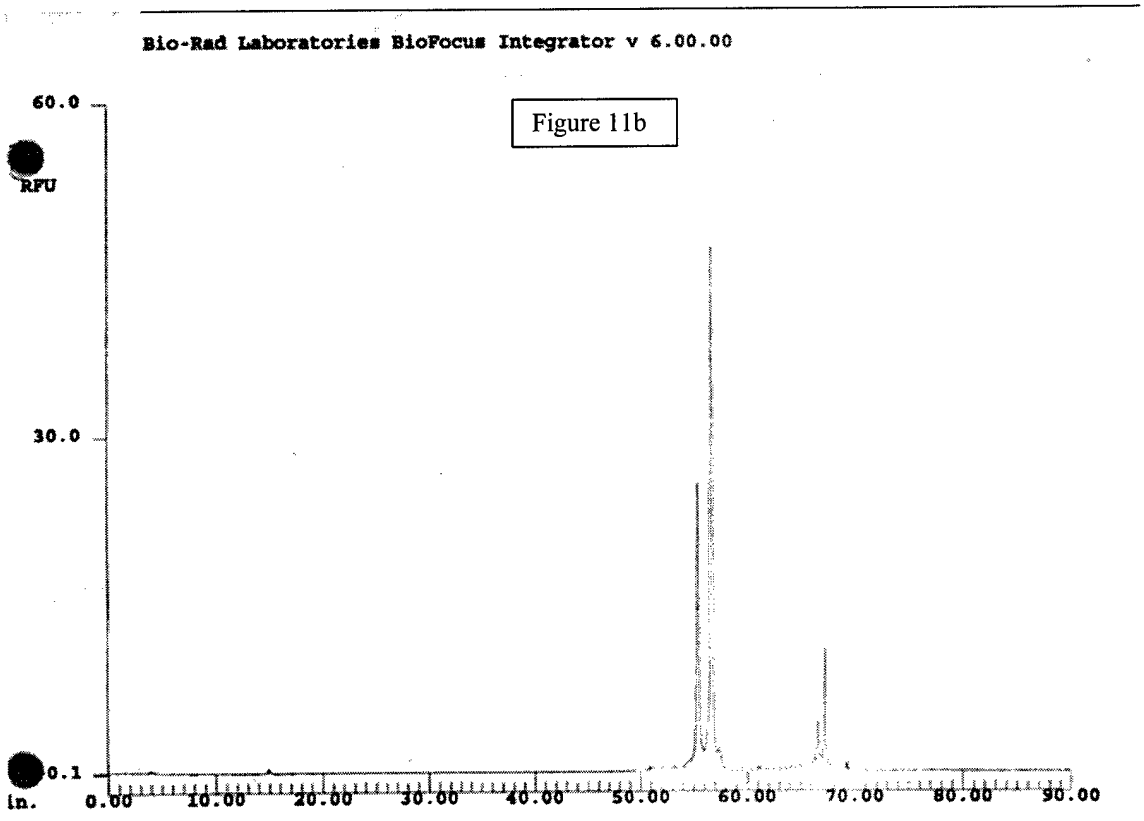


Figure 11: 6 % HMW PVP/10 % Methanol /X TBE with SYBR® Green at 1.5 kV 100 (psi x sec)

Figure 11a: TRAP PCR Product distilled water blank: primer-dimer peak
Figure 11b: TRAP PCR Product ITAS and primer-dimer peaks

Results show a distinct primer-dimer peak, which was significantly larger than the ITAS peak. In order to confirm the smaller peak indeed represented the ITAS product, an experiment was performed in which half the amount of primers was used during PCR amplification (Figure 11c). Results confirmed the ITAS location, as a decrease in the primer-dimer peak and an increase the ITAS peak occurred simultaneously.

Preliminary data show the separation and identification of the TRAP assay bands can be performed using 6 % HMW PVP in 10 % methanol with 100 (psi x sec) injection times at 1.5 kV, and suggests subsequent quantitation of the assay is possible.

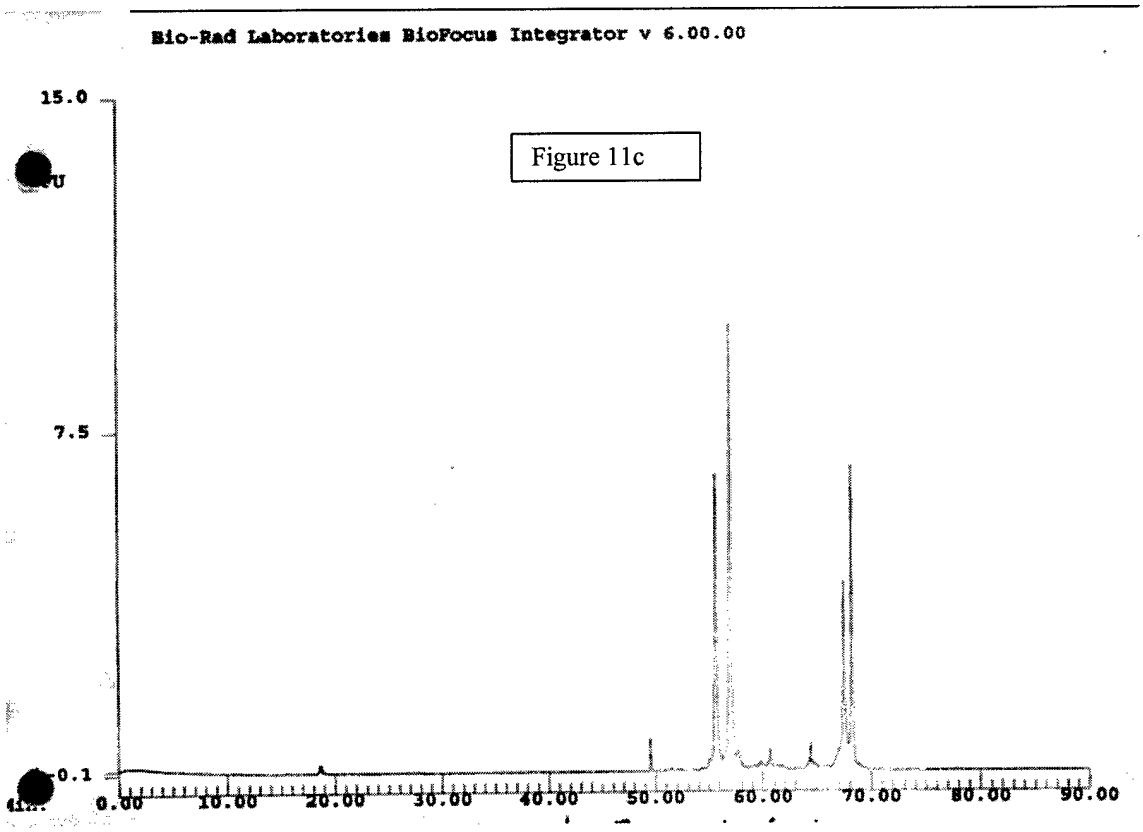


Figure 11c

Figure 11: 6 % HMW PVP/10 % Methanol /X TBE with SYBR® Green at 1.5 kV 100 (psi x sec)

Figure 11b: TRAP PCR Product ITAS and primer-dimer peaks with half the amount of primers in both MMA and MMB

DICUSSION

Telomeres are specialized structures that protect the ends of eukaryotic chromosomes from enzymatic degradation and help to maintain genetic stability. Because of the inability of DNA polymerase to fully replicate the ends of linear DNA, telomeres become progressively shorter with each cell replication. Eventually, a critical point is reached at which replication can no longer occur and the cell goes into senescence (Shay 1997). Telomere shortening is thought to be a molecular mechanism that limits the replicative ability of the cell and is a natural part of cellular aging (Harley 1991, Shay 1997).

Telomerase is a ribonucleoprotein enzyme that compensates for telomeric shortening by adding hexameric repeats (TTAGGG) to the ends of telomeric DNA during cell replication (Shay 1997). This prevents critical shortening, thereby avoiding cellular senescence, and essentially immortalizing the cell. Telomerase is normally present in embryonic cells, germline cells, and renewal tissue such as hematopoietic stem cells, activated lymphocytes, basal cells of the epidermis, and intestinal crypt cells (review by Hiyama *et al* 1995a). Telomerase activity has been isolated in the tissue of 85-95 % of all tumor types tested, but has been undetectable in normal somatic cells (Shay 1997). The high prevalence of telomerase activity in tumor tissue, and its relative absence in somatic tissue, would make it ideal as a universal tumor marker.

The importance of identifying such a tumor mark is that by the time patients present with clinical symptoms the cancer is already in advanced stages and metastases may have already occurred (Kohn and Liotta 1995). Despite improvements in the early diagnosis and treatment of cancer, many deaths occur due to the spread of malignant cells that fail to respond to therapy. If treatment could be started when the tumor burden is still small and before metastasis has the chance to occur, survival rates could potentially be increased. The prostate-specific antigen (PSA) is an excellent example of a tumor marker that is specific, easily detectable in the blood, enhances accurate staging of disease, and can be used for monitoring response to treatment (discussion by Strovel 1999). Early detection allows for early intervention, ultimately increasing chances of survival. Other tumor markers have been investigated for screening various cancer types, and while some, such as CA 125 and carcinoembryonic antigen (CEA) are useful in monitoring therapy response, they lack the specificity and sensitivity necessary for screening purposes. If a method could be developed to detect telomerase activity in the blood, as in the case of PSA, it could prove to be very beneficial in the early diagnosis of cancer.

Jeffrey Strovel, Ph.D., Division of Human Genetics, University of Maryland, Baltimore, successfully modified the TRAP assay, initially developed by Kim *et al* (1994), to detect telomerase activity from the blood of lung cancer patients. Stovel's data showed 70 % sensitivity for NSCLC patients, and 100 % sensitivity for SCLC patients which correlated extremely well with data obtained by

Hiyama *et al* (1995b) who obtained 73 % and 100 % sensitivity, respectfully, when testing biopsied tissue specimens from NSCLC and SCLC patients. This remarkable concordance between the positivity rates seen in tumor tissue and in blood suggests that measurement of telomerase activity in blood accurately reflects the telomerase status of the patient's tumor.

The TRAP assay is a PCR based procedure that utilizes a radioactive isotope for the detection and quantitation of telomerase activity. The PCR products are electrophoresed on a polyacrylamide gel followed by autoradiography or PhosphorImager analysis. While useful in a research setting, this procedure is time-consuming, cannot be automated, uses hazardous radioisotope, and is unsuitable for high-throughput analysis or utilization in a clinical laboratory setting.

Capillary electrophoresis with laser induced fluorescence (CE-LIF) is a highly sensitive adaptation of electrophoresis that offers automation, rapid separation, on-line detection, and extremely high resolution for both small and large molecules. The use of narrow diameter capillaries in electrophoresis increases the surface to volume ratio of the capillary which allows for the efficient dissipation of Joule heat generated from the applied electrical field. This permits the use of high electric fields, increasing the speed of analysis and resulting in high resolution separations. The small bore capillaries also greatly decreases the volumes of sample and reagents required for analysis. Laser induced fluorescence not only

increases the sensitivity, but also obviates the need of using a radioactive isotope for detection and quantitation. CE-LIF has the potential of automating the quantitation of the TRAP assay product in a high-throughput, user friendly manner, that is less time consuming than the current method, without the use of a radioisotope.

The purpose of this thesis is to optimize a CE-LIF platform that is automatable, has the requisite sensitivity, does not utilize radioisotopes, is user friendly, and is exportable to a clinical laboratory setting, for the analysis of telomerase activity.

Initial separations were achieved using the water soluble linear polymer polyvinylpyrrolidone (PVP) at concentrations between 5 %-7.5 %. By reducing the voltage to 1.0 kV, resolution at a 25bp level was achieved. For separation of smaller DNA fragments, the technique of Gao and Yeung (1998) was used for fractionating high molecular weight (HMW) PVP, and separation of TRAP PCR products at a 6bp level was achieved using a 6 % HMW PVP. 10 % methanol was added to the 6 % HMW PVP to test the effect it had on viscosity and resolution. Resolution did seem to increase while slightly decreasing the migration time. However, additional testing would need to be performed to determine if the resolution increase was significant over that of 6 % HMW PVP without methanol. Separation of K562 PCR product at a 25 cell level was achieved using 6 % HMW PVP in 10 % methanol at 1.5 kV.

Molecules will migrate faster in a lower ionic strength solutions, and this can be used in CE to concentrate like molecules prior to their migration through the buffer. The PCR specimens were diluted 5X to achieve one-tenth the ionic strength of the sieving matrix, and stacking effects were determined by increasing the sample injection times. This increased the sample amount in the capillary (sample plug), and allowed the molecules more time to line up (stack) prior to migration. In essence, this serves to concentrate the sample prior to electrophoresis. Dramatic effects were achieved using a ten base pair ladder, and even though the laser was in decay, the detection limit was exceeded when stacking was performed at 70 (psi x sec). While results were not as dramatic in the TRAP PCR 6bp ladder, sensitivity was still increased two fold. Additional testing needs to be done to determine maximum sample loading without loss of resolution.

Having successfully separated the TRAP PCR ladder, the next step was to determine the migration peaks of the primer-dimer band and the ITAS band. 6 % HMW PVP in 10 % methanol was used, samples were injected for 100 (psi x sec) and electrophoresis was performed at 1.5 kV. Using distilled water, the primer-dimer peak was easily detected, and although there was a peak representing the ITAS fragment, it was significantly smaller when compared with the primer-dimer peak. To enhance the ITAS peak, the amount of primers were reduced by half in both MMA and MMB. The effect was to decrease the height of the primer-dimer peak, while simultaneously increasing the height of the ITAS peak, confirming the identity of the ITAS peak.

Preliminary data of these experiments identify the conditions for the separation and identification of the primer-dimer, ITAS, and telomerase TRAP ladder fragments. Subsequent testing confirming enhanced separation with the addition of methanol to 6 % HMW PVP, the maximum load of detection, and the linearity of the procedure, would further optimize the conditions identified by these experiments. In addition, correlation studies comparing CE quantitation of TRAP assay products with radioactive quantitation need to be done to confirm the utility of using CE-LIF over radioactive PAGE. With the development of Capillary Array Electrophoresis (CAE) which simultaneously separates 96 samples using laser induced fluorescence, the results of these experiments could be used to develop a rapid, high-throughput method for quantitating the TRAP assay in clinical setting.

Additional testing is currently in progress at UM, B extending Strovel's study of testing blood samples to include other tumor types not only for screening purposes, but also for correlation of treatment with regards to therapy response, remission, and relapse. The current methodology is time consuming, tedious, not adaptable to automation, and uses a radioactive isotope. This research has shown that CE-LIF can be used to separate TRAP PCR products and differentiate between the primer-dimer, and the ITAS fragments for quantitation of telomerase activity. With further testing, and CAE technology, CE-LIF has the potential of being used to accelerate the process of testing the many different tumor types that exhibit telomerase activity and answering the questions of the utility of using telomerase as

a tumor marker and prognostic tool to aid the clinician in increasing the survival rate for cancer patients. Studies are also underway to evaluate different methodologies for the detection of telomerase, some of which do not rely on the activity of the enzyme, which degrades rapidly under physiological conditions. While future testing will likely include immunoassays that will be far easier and user friendly, there is still much research to be done before immunoassay applications will be available. CE-LIF could provide a more rapid, safer, cost effective means of obtaining the answers necessary to advance research.

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